

**STUDIES ON THE INTEGRATED CONTROL OF THE RUSSIAN
WHEAT APHID, *DIURAPHIS NOXIA* (KURDJUMOV) (HEMIPTERA:
APHIDIDAE), USING ENTOMOPATHOGENIC FUNGI COMBINED
WITH SUBLETHAL DOSES OF INSECTICIDES**

By

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Dissertation Summary

The Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov) (Hemiptera: Aphididae), is one of the most damaging aphid pests of wheat grown under dryland conditions. Host plant resistance is considered as the most, cost-effective, and ecologically attractive method of control for this cereal pest. However, it is a slow process to breed wheat varieties for resistance to RWA, and resistance-breaking biotypes continue to pose a serious threat to wheat producers in the country. Currently, there are four RWA biotypes that have been recorded in South Africa.

Entomopathogenic fungi (epf), such as *Beauveria bassiana*, have the potential to suppress RWA as shown by Hatting *et al.* (2004), whereby *B. bassiana* together with host plant resistance managed to provide about 60% level of control of this pest. Nevertheless, that was not a satisfactory level of control. Furthermore, hyphomycetes are known to kill their target insects more slowly than chemicals. One approach to increase their efficiency, and therefore insect mortality is by combining epf with sub-lethal doses of chemical insecticides. The interaction observed from this combination can be synergistic, antagonistic or neutral. Synergistic effects would allow for reduced insecticide use, minimized environmental pollution, the preservation of natural enemies and a slowing of the development of insecticide resistance. The hypothesis for the synergistic interaction is that the insecticide acts as a stress inducer, making the insect pest more susceptible to fungal infection.

Given the development of new resistance-breaking biotypes of the RWA and many reported cases of insecticide resistance, this study aimed to enhance the virulence of selected entomopathogenic fungal strains through synergism with sub-lethal doses of chemical and botanical insecticides.

The first objective was to find several virulent entomopathogenic strains of *B. bassiana* and *M. anisopliae* against *D. noxia*. The virulence of three *Beauveria bassiana* and three *Metarhizium anisopliae* strains were evaluated against *D. noxia* biotype RWASA1. *B. bassiana* Isolate SG1921, was the most pathogenic strain, and was used for the subsequent studies.

Four insecticides registered for RWA control were screened *in vitro* for compatibility with the selected fungal strain, SG1921, with special emphasis on germination, radial

vegetative growth and sporulation intensity. All the tested insecticides (active ingredients: chlorpyrifos, dimethoate, demeton-S-methyl and acetamiprid) reduced germination, radial vegetative growth and sporulation intensity in various degrees in a concentration-dependent manner. Mospilan was found to be compatible with the selected strains and, at its highest concentration, it seemed to have stimulated vegetative growth.

The last objective was to determine the effect of combining sub-lethal doses of pyrethrum-based insecticides with either a commercial formulation of *Beauveria bassiana*, Eco-Bb®, and *Beauveria bassiana* Strain R444 in both *in vivo* and *in vitro* trials. In the laboratory bioassays 10% Pyrol was able to enhance the sporulation intensity of R444. In glasshouse trials 10% Pyrol enhanced the efficacy of *B. bassiana* Strain R444 by increasing RWA mortality and reducing the mean time to mortality. At a concentration of 10%, Mospilan killed the aphids before either of the fungi could infect the aphids, *i.e.*, within 48 hours post fungal inoculation. Mospilan concentrations below 10% will be further investigated to establish a minimal sub-lethal dose that does not kill the RWA but make the aphids more susceptible to epfs. The interaction of Pyrol with the unformulated strain of *B. bassiana* Strain R444 will be tested in other biotypes of Russian wheat aphids. Since vegetable oils are also known to enhance efficacy of entomopathogenic fungi, future research will also evaluate the interactions of the pyrethrum extract and canola oil, in order to enhance the synergistic effects of the oil in the Pyrol and *B. bassiana* interaction.

Declaration

I, **Nokulunga Prudence Mzimela**, declare that

- i) The research reported in this thesis, except where otherwise indicated, is my original work.
- ii) This dissertation has not been submitted for any degree or examination at any university.
- iii) This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being from other persons.
- iv) This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers.

Where other written sources have been quoted:

- (a) Their words have been re-written but the general information attributed to them has been referenced;
- (b) Where their exact words have been used, their writing has been placed inside quotation marks, and referenced.

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“I can do all things through him who strengthens me“. Philippians 4:13

Dedication

This dissertation is dedicated to my parents, *Mr and Mrs T.P Mzimela*, and my daughter, *Kwandokuhle*, for the love and support throughout this journey.

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Dissertation Introduction

Wheat is the most commonly cultivated cereal in the world, with more than 220 million hectares planted annually in many geographic locations (Shiferaw *et al.*, 2013). About 10 million hectares are planted annually in Africa (MacAulay, 2015). It is one of the most important crops for global food security and provides 20% of the daily protein and of the food calories to about 4.5 billion people (GCARD, 2012). However, current wheat production levels do not satisfy demand already. With the world population expected to grow to 9 billion by 2050, wheat demand is expected to increase by 60% in the next 30 years (GCARD, 2012).

South Africa is a net importer of wheat and depends on imports to supply the growing local demand of wheat (SAGL, 2016). Although, the area planted with wheat increased slightly by 1.2% in the 2015/2016 growing season, the mean national yield declined from 3.67 t ha⁻¹ (2014/2015) to 2.99 t ha⁻¹ (2015/2016). The quantity of commercial wheat produced in the 2015/2016 season was also 17.7% lower than the previous season (SAGL, 2016). The net result is that South Africa imports increasing quantities of wheat annually.

It is difficult for South African farmers to produce wheat profitably and they have slowly switched to more profitable crops such as canola, maize and soybean (USDA, 2013). Abiotic (aluminium toxicity and pre-harvest sprouting) and biotic stresses (insect pests and pathogens) are the primary reason for the poor wheat yields. Russian wheat aphid is the most injurious wheat pests, and has caused yield losses of about 60% on susceptible South African cultivars (Du Toit and Walter, 1984).

Wheat planted in dryland conditions is highly susceptible to the most damaging aphid pest, Russian wheat aphid (RWA), *Diuraphis noxia* (Kovalev *et al.*, 1991; Turanli *et al.*, 2012). Breeding for RWA resistance is regarded as the most environmentally safe approach to manage this cereal pest (Tolmay, 2006, Pathak *et al.*, 2007; Porter *et al.*, 2009). Wheat producers are constantly threatened by resistance breaking biotypes (Jankielsohn, 2011). Agricultural Research Council-Small Grain, introduced an IPM programme against this pest, with the approach being based on breeding for RWA resistance and use of natural enemies.

From that programme, formulated *Beauveria bassiana* Strain GHA, proved the ability to manage RWA where control levels were about 65%. The major hindrance factor was that, wheat farmers expected a much higher level of control. However, a higher level of inoculum may be required to achieve that desired control (Boucias *et al.*, 1996). An alternative approach to enhance the levels of insect mortality is to combine epf strains with sub-lethal doses of chemical or botanical insecticides. The consequent interactions can be synergistic, antagonistic or neutral (Sahoo and Dangar, 2014). Achieving synergistic interactions could cause a reduction in insecticide use, minimized environmental pollution, and a reduction in the development of insecticide resistance (Ambethgar, 2009). The insecticide induces stress on the insect, making it more susceptible to fungal infection (Benz, 1971; Ambethgar, 2009; Malekan *et al.*, 2012).

This study aimed to enhance the virulence of selected entomopathogenic fungal strains through synergism with sub-lethal doses of chemical and botanical insecticides.

The objectives of the study were:

- To find several virulent entomopathogenic strains of *B. bassiana* and *M. anisopliae* against *D. noxia*
- To screen insecticides, registered for RWA control, for *in vitro* compatibility with selected fungal strains in terms of germination, radial vegetative growth and sporulation.

To determine the effects of combining sub-lethal doses of pyrethrum-based insecticides with a commercial formulation of *Beauveria bassiana*, Eco-Bb[®], and an unformulated strain of *Beauveria bassiana* Strain R444.

The thesis has four chapters, where Chapter 1 is the literature review discussing methods to enhance efficacy of entomopathogenic fungi, with special emphasis on *Beauveria bassiana*, and Chapters 2, 3 and 4 are the experimental chapters. Each chapter was derived from the objectives that are stated above.

Chapter 2 was presented as a speed presentation at the joint XIX Entomological Society of Southern Africa and the 37th Zoological Society of Southern Africa congress in Grahamstown, South Africa in 2015. Chapter 3 was presented in poster format at the International Congress of Entomology in Orlando, Florida, USA in 2016. Chapter 4 will be presented at the African Combined Congress in Cape Town, in 2018.

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Chapter 1

A literature review

1.1 Introduction

Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov) (Homoptera: Aphididae), is one of the most destructive aphid pests of wheat grown under dryland conditions (Kovalev *et al.*, 1991; Turanli *et al.*, 2012); and can reduce individual plant yield by 90% (Du Toit and Walters, 1984). Chemical insecticides were the first and most effective method of control used against this pest. However, development of resistance, environmental pollution, decrease in biodiversity, chemical residues on the harvested grain and the high-costs associated with the use of chemical insecticides has led to a greater focus on the development of other control methods (Kandpal, 2014). Breeding for resistance has been considered the most cost-effective and sustainable management practise against RWA, with the first resistant cultivar (Tugela-DN) released in 1992 in South Africa (Van Niekerk, 2001; Tolmay, 2006; Porter *et al.*, 2009). Subsequently, the wide spread use of resistant cultivars has led to increased selection pressure, resulting in the first resistance-breaking biotype (designated RWASA2) reported in 2005 (Tolmay *et al.*, 2007). Since then, a total of four RWA biotypes have been recorded in South Africa (Jankielsohn, 2014a). The Small Grain (SG) of the Agricultural Research Council (ARC-SG) promotes the integration of other strategies with host plant resistance for control of RWA, *i.e.*, biological control using natural enemies such as parasitoids, predators and entomopathogens (Marasas *et al.*, 1997; Hatting, 2002).

The natural enemies associated with *D. noxia* in South Africa include the predators, coccinellid beetle *Adonia variegata* Goeze, *Sycmnus morelleti* Mulsant, paraitoids, *Aphidius colemani* Viereck, *Aphelinus asychus* Walker, *Aphelinus hordei* Kurdjumov, and entomopathogenic fungi, *Pandora neoaphidis* (Remaudière & Hennebert) Humber, *Conidiobolus obscurus* (Hall & Dunn) Remaudière & Keller, *C. thromboides* Drechsler, *C. coronatus* (Costantin) Batko, *Entomophthora planchoniana* Cornu, *Neozygites fresenii* (Nowakowski) Remaudière & Keller, *Beauveria bassiana* (Balsamo) Vuillemin and *Lecanicillium lecanii* (= *Verticillium lecanii*) (Zimmermann) Gams & Zare (Aalbersberg *et al.*, 1984, 1988a; Prinsloo, 1990, 1998; Hatting *et al.*, 1999; Hatting, 2002). However, studies conducted in South Africa revealed that natural

enemies attacking *D. noxia* under dryland conditions in the summer rainfall region were unable to contain this aphid below injury levels (Aalbersberg, 1987; Aalbersberg *et al.*, 1988a; Prinsloo, 1990).

Key reasons include the low thermal threshold for *D. noxia* nymphal development of only 0.54°C (Aalbersberg *et al.*, 1987), enabling South African populations of *D. noxia* to rapidly increase in numbers during the early wheat season (*i.e.*, September onwards, when density-dependant enemy numbers are still low (Greathead, 1992). Moreover, reduced soil water content during early spring cause stressed plant populations (Prinsloo *et al.*, 1995), which compound the problem, in favour of the aphid. Entomopathogenic fungi (epf) in the Clavicipitaceae have been found to naturally infect *D. noxia* in South Africa (Hatting *et al.*, 2000), indicating the potential to suppress this pest, but have not been found to cause natural epizootics as do members of the Entomophthoromycota. The potential of entomophthoralean fungi in controlling cereal aphid populations was highlighted by Hatting *et al.* (2000). Numerous studies have indicated the potential of using entomopathogenic fungi for the management of cereal aphids (Feng *et al.*, 1990a; Mesquita *et al.*, 1996; Vandenberg, 1996; Vandenberg *et al.*, 2001; Hesketh *et al.*, 2008; Ganassi *et al.*, 2010; Sedighi *et al.*, 2012; Akmal *et al.*, 2013, Fekih *et al.*, 2013; Fadayivata *et al.*, 2014; Murerwa *et al.*, 2014a).

Beauveria bassiana and *Metarhizium anisopliae* (Metschnikoff) Sorokin affect many different insect hosts (Butt *et al.*, 1994) and are currently used for the management of various insect pests, worldwide (de Faria and Wraight, 2007). *Beauveria bassiana* has been applied on cereal aphids under glasshouse conditions: *D. noxia*, *Schizaphis graminum* Rondani, *Metopolophium dirhodum* Walker, *Sitobion avenae* Fabricius, *Rhopalosiphum maidis* Fitch and *Rhopalosiphum padi* Linnaeus (Feng *et al.*, 1990a); *D. noxia* (Vandenberg, 1996); *S. graminum* and *R. padi* (Akmal *et al.*, 2013); and field conditions, *D. noxia* (Vandenberg *et al.*, 2001; Hatting *et al.*, 2004).

Nonetheless, only a few reports are available where *M. anisopliae* has been applied against cereal aphids (Hesketh *et al.*, 2008, Murerwa *et al.*, 2014a; 2014b). This might be because *M. anisopliae* has not been found to infect any cereal aphid in recorded field surveys of entomopathogenic fungi infecting cereal aphids in South Africa (Hatting *et al.*, 1999) and China (Feng *et al.*, 1990b).

Species such as *B. bassiana* and *M. anisopliae* are easy to formulate, show considerable shelf-life, are distributed in most regions of the world and can be easily isolated from insects and soil (Meyling *et al.*, 2006, Freed *et al.*, 2011). However, in a field trial with an isolate of the fungus *B. bassiana*, combined with host plant resistance against RWA, caused levels of control that ranged from 60-65% (Hatting *et al.*, 2004). The desired levels of control for RWA was not achieved, but highlighted that *B. bassiana* has the potential to be used as one component of integrated pest management (IPM). The fungus may be slow to cause sufficient insect mortality, and inconsistent performance under field conditions, which are major limitations in the use of hyphomycetes (Sharififard *et al.*, 2011).

In the eastern Free State, where RWA is the most damaging insect pest, wheat is planted from mid-May up to the end of July, begins to ripen mid-November and is harvested during December (Aalbersberg *et al.*, 1988b). Other biotic stresses of importance include stripe rust (*Puccinia* Westend f. sp. *striiformis* Eriks.), leaf rust (*Puccinia triticina* Eriks.) and crown rot (*Fusarium pseudograminearum*) (Tolmay, 2006).

1.2 Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov) (Homoptera: Aphididae)

Adults are small, approximately 1.6 to 2 mm long, lime-green in colour, and spindle-shaped (Figure 1.1). This wheat pest is characterised by shortened legs, antennae and cornicles (Stoetzel, 1987; Hodgson & Karren, 2008). The side view shows that the terminal segment of the abdomen has the supracaudal structure that resembles a 'double tail' (Du Toit and Aalsbersberg, 1980; Stoetzel, 1987).



Figure 1.1: Russian wheat aphid, *Diuraphis noxia* (JL Hatting, ARC-SG).

1.2.1 Origin and Distribution

Kovalev *et al.*, (1991) stated that the very first appearance of *D. noxia* was in 1901, in the Crimea. The Russian wheat aphid (RWA), *Diuraphis noxia* (Mordvilko) is native to the steppe region of southern Russia, Iran, Afghanistan and countries bordering the Mediterranean sea (Kiriak *et al.*, 1990; Dolatti, *et al.*, 2005; Ennahli *et al.*, 2009). In 1912, sporadic outbreaks of RWA occurred in the former Union of Soviet Socialist Republic (USSR), where losses of about 75% were reported (Halbert and Stoetzel, 1998). From there it has spread rapidly to South America, USA and Canada and most parts of Europe (Pathak *et al.*, 2007). This damaging pest now occurs in almost all wheat and barley producing areas of the world and the first occurrence in Australia was confirmed in May 2016 (Baker *et al.*, 2017).

In Africa, RWA was first reported in Adigrat regions of Ethiopia in the early 1970s (Adugna and Megenasa, 1987); by 1976 it had spread to all barley and wheat growing areas in that region (Haile, 1981). In 1978, *D. noxia* was first reported in South Africa as a pest of wheat (Walters, 1980). By 1980, RWA was discovered in central Mexico and in late 1980's the aphid's infestations had reached southern Texas in the United States of America (Stoetzel, 1987). Pathak *et al.* (2007) indicated that RWA is still a minor pest in Egypt, Sudan and Ethiopia but invaded Kenya in 1995, where it is known as the most important pest of wheat and barley. The Russian wheat aphid is responsible for significant losses in wheat in USA, South Africa and South America (Liu *et al.*, 2002).

1.2.2 Biology and Life cycle

In South Africa, all RWA produced are females, which reproduce asexually throughout the year, and each give birth to live daughters carrying embryonic granddaughters (telescoping generations) (Aalbersberg, *et al.*, 1987). This method of reproduction is known as parthenogenicity. Aphids are typically r-strategists, having the ability to increase rapidly because of parthenogenic reproduction, with a short generation time (Hales *et al.*, 1997, Akmal *et al.*, 2013) and an efficient dispersal strategy (Harrewijn, 1997). Ricci *et al.* (2011) noted that the reproductive strategies adopted by RWA have a significant influence on the production of new biotypes, which can overcome the current resistance gene/s in wheat cultivars.

Russian wheat aphid feed on wheat until the plant gets to maturity, and can also be found in developing heads (Tolmay, 2006). When the wheat plant is under stress in response to heavy aphid feeding or physiological maturity, an increased proportion of immature aphids develop wings known as the alate form (Walters *et al.*, 1980). *Diuraphis noxia* populations vary with season, between geographic locations and from year to year, and will increase when conditions are conducive for reproduction (Jankielsohn, 2009; Chapela, 2013).

Temperature is the most important factor that determines the rate of development, fecundity and lifespan of the aphids (Aalbersberg *et al.*, 1987). Aalbersberg *et al.* (1987) found that an increase in temperature could result in increased nymph production and a decrease in the lifespan of RWA. Generation times range from 8 to 42 days (Aalbersberg *et al.*, 1987). Porter *et al.* (2009) found that females can produce 13 to 46 nymphs per generation. Lastly, high temperatures induced rapid development and early production of offspring. Therefore, outbreaks of *D. noxia* can be greatly accelerated by favourable temperatures (ranging from 10°C to 20°C) (Aalbersberg *et al.*, 1987).

1.2.3 Host plants

The Russian wheat aphid attacks a wide range of plant species within the *Poacea* family, including wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) (Halbert and Snoetzel, 1998). When cereal crops such as wheat and barley are not available, wild grasses can serve as the alternate host for RWA (Kindler and Springer 1989; Jankielsohn, 2011; Turanli *et al.*, 2012). For a RWA to survive in a region it must have a host that grows all year round. Neglected volunteer wheat can be important to RWA survival by serving as an alternate host. The absence of wheat between the period of harvest and next crop emergence in late winter forces this pest to migrate to alternate hosts (Jankielsohn, 2009; Porter *et al.*, 2009). Volunteer wheat (Aalbersberg *et al.*, 1988b), wheat regrowth, wild oats (*Avena fatua*), rescue grass (*Bromus catharticus*), canary grass (*Phalaris minor*) and false barley (*Hordeum murinum*) are the alternate host plants for RWA in South Africa (Jankielsohn, 2009) and they can host the pest until the following season. A survey that was conducted in the eastern Free State by Aalbersberg *et al.* (1988b) indicated that *Bromus* and volunteer wheat played an important role in the survival of *D. noxia* between seasons.

1.2.4 Symptoms and damage

Diuraphis noxia attacks the plant by infesting the young growing tip, deep in the leaf whorls where it feeds from the phloem of longitudinal veins (Masinde *et al.*, 2014). Nymphs and adults feed on plant phloem with a piercing-sucking stylet. Russian wheat aphids feed on foliage and grain spikes of actively growing plants, with the most susceptible stage to RWA damage being the period from flag leaf appearance to flowering (Du Toit and Walters, 1984).

The RWA causes direct and indirect damage in wheat plants (Pathak *et al.*, 2007). Direct damage occurs when the aphids feed in leaf whorls and suck sap from the affected plants, whereas indirect damage occurs when a toxin is injected into the wheat plant (Pathak *et al.*, 2007). The injected toxin causes distortion and discolouration of the plant (El Bouhssini *et al.*, 2011). The damage is characterised by longitudinal white, yellow, or red chlorotic streaks, accompanied with leaf rolling (Figure 1.2; Marasas *et al.*, 1997; Tolmay and Mare, 2000; El Bouhssini *et al.*, 2011), which reduces the photosynthetic area but protects the aphid against natural enemies and contact insecticides (Khan *et al.*, 2010; El Bouhssini *et al.*, 2011). The effect of indirect damage can result in a reduction of plant height, sterile heads, and/or low kernel weight (Walters, 1984; Masinde *et al.*, 2014).

The signs and symptoms of RWA infestations are very distinct (Marasas *et al.*, 1997). Walters *et al.* (1980) observed that heavy infestations in young plants cause tillers to become prostrate whereas in the later growth stages ears become trapped in the rolled flag leaf. Furthermore, Burd and Burton (1992) found that RWA infestation also caused water imbalances, and reduced growth, resulting to considerable loss in biomass. If the flag leaf is infested and curls at the same time when the head emerges, the head may become trapped, thus giving a fish-hook appearance (Figure 1.2).

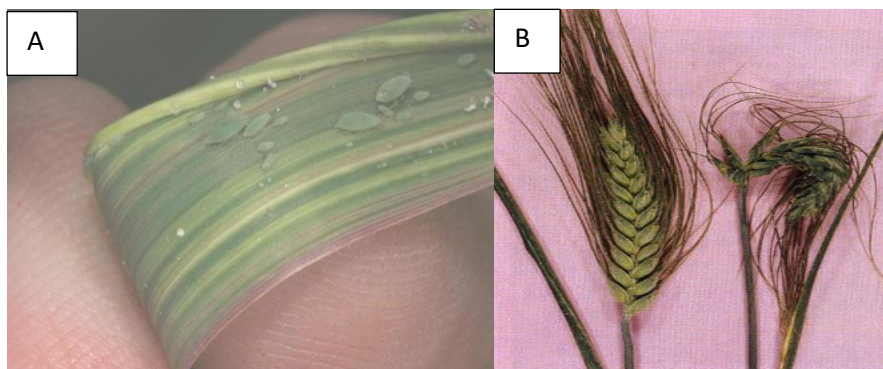


Figure 1.2: Heavy infested wheat plants showing various symptoms, (A) longitudinal yellow and red chlorotic streaks and, (B) fish-hook appearance (WAAESD, 2012).

1.3 Management strategies for RWA

1.3.1 Host plant resistance

Host plant resistance is regarded as the most desirable (Masinde *et al.*, 2014); sustainable, cost-effective, and ecologically attractive method of control (Tolmay, 2006, Pathak *et al.*, 2007; Porter *et al.*, 2009), because planting RWA-resistant wheat cultivars reduces the need to use other control methods such as chemicals (El Bouhssini *et al.*, 2011). The first South African cultivar resistant against RWA (Tugela-Dn) was released in 1992 (Van Niekerk, 2001; Marasas *et al.*, 2005). The occurrence of RWA Biotypic variation after the deployment of RWA resistant cultivars in South Africa raised concerns about the future durability of host plant resistance (Jankielsohn, 2014b). A new biotype, designated RWASA2, was identified in 2005, which is virulent against the *Dn1* resistant gene in wheat (Tolmay *et al.*, 2007). A third biotype (RWASA3) that is virulent against the *Dn4* resistant gene in wheat, was recorded in 2009 by Jankielsohn. (2011). In 2011 RWASA4 which is virulent against the *Dn5* resistant gene was recorded near Bethlehem in the Eastern Free State (Jankielsohn, 2014a). Development of virulent RWA biotypes continue to pose a serious threat to wheat producers in the country (Jankielsohn, 2011).

1.3.2 Chemical control

There are several contact and systemic insecticides registered for the control of RWA (NDA, 2007). These are broad-spectrum neonicotinoids, organophosphates and carbamates (Nel *et al.*, 2002; van Zyl, 2013). A guide for the control of plant pests listed chlorpyrifos, demeton-S-methyl, dimethoate and acetamiprid as registered aphicides for RWA (van Zyl, 2013). Organophosphates and carbamates are acetylcholinesterase

inhibitors. They inhibit the transmission of nerve impulses across the synaptic gap between two nerve cells (Tomizawa and Casida, 2003). Neonicotinoids (heterocyclic nitromethylenes) are systemic pesticides known to have less toxicity than previously used organophosphates and carbamates (Chao and Casida, 1997). These systemic pesticides are currently used against sap-sucking insects, including cereal aphids. Systemic insecticides are absorbed by plant tissues and protect the plants from the inside out, because the insects ingest the chemical while feeding on the plant. Acetamiprid, imidacloprid and thiamethoxam are commonly used insecticides from the group (Neves *et al.*, 2001). They have the same mode of action as nicotine. This is the linking of synapses of the nervous connections to the acetylcholine receptors, resulting in paralysis, which is followed by death (Elbert *et al.*, 1991; Tomizawa and Casida, 2003).

Contact insecticides are sprayed on the surface and toxins are absorbed through the cuticle and they are often ineffective due to the aphid's characteristic habit of hiding within a rolled leaf (Ennahli *et al.*, 2009). Other countries have banned the use of organochlorides, organophosphates and carbamates since they are toxic to non-target insects such as bees. Indiscriminate use of chemicals in the wheat industry should be of great concern because of insecticide resistance and other food safety issues. IPM components such as myco-insecticides are under-utilized because of an established dependence on chemicals. Entomopathogen-based bioinsecticides have not been very successful because of the high fecundity of cereal aphids.

1.3.3 Parasitoids and predators

The use of natural enemies such as parasitoids and predators to manage *D. noxia* is part of IPM strategy in almost all wheat growing regions (Brewer and Elliot, 2004). In a survey conducted in South Africa by Aalbersberg *et al.* (1988a) in the early 1980's the coccinellid *Hippodamia variegata* Goeze and two braconids *Diaeretiella rapae* M'Intosh and *Aphidius colemani* Viereck were the most prevalent natural enemies and were closely associated with *D. noxia*. The population increase of these natural enemies is lower than that of RWA (Adisu *et al.*, 2003), and therefore they are unable to keep the aphids below the economic threshold. Indigenous natural enemies such as ladybirds were found to be ineffective in protecting the susceptible cultivars from aphid damage in SA (Aalbersberg *et al.*, 1988a). As part of a classical biological control programme, natural enemies of *D. noxia* were introduced from countries where the pest originated

in order to achieve higher levels of control. Prinsloo (2006) reported six natural enemies that were introduced and released between 1980 and 1994. Those were the coccinelids *Adalia bipunctata* Linnaeus, *H. convergens* and *Coleomigilla maculate* De Geer, the silver fly *Leucopis ninae* Tanasijtshuk, the braconid wasp *Aphidius matricariae* Haliday, and aphelinid wasp *Aphelinus hordei* Kurdjumov.

However, only three established locally. The recovery of *A. hordei* had to be verified by polymerase chain reaction because the species is very similar in colour and structure to *A. varipes* Foerster and *A. albipodus* Hayat & Fatima (Prinsloo *et al.*, 2002). Additionally, most of the natural enemies of RWA are polyphagous (Marasas *et al.*, 1997), and their numbers increase when the crop is heavily infested with the RWA and by that time the crop is already stressed. The ineffectiveness of the existing complex of parasitoids and predators in controlling RWA populations underscores the need of employing other biocontrol strategies (Wraight *et al.*, 1993) and biopesticides have promising potential (Valero-Jimenez *et al.*, 2016).

1.4 Entomopathogenic fungi as microbiological control agents

Microbiological control agents are an environmentally responsive substitute for chemical insecticides (Khan *et al.*, 2012). Entomopathogenic fungi comprise of a heterogeneous group of over 100 genera with about 750 species reported from different insects (Alice *et al.*, 2014). Fungi are important natural regulators of many arthropods (Shah and Pell, 2003; Ambethgar, 2009; Asi *et al.*, 2010; Khan *et al.*, 2012), including several major insect pests (Carruthers and Hural, 1990; Amutha *et al.*, 2010). Their efficacy is mainly due to their pathogenicity, broad host range and their ability to manage both sap sucking pests as well as pests with chewing mouth parts (Khan *et al.*, 2012). More than 700 species of fungi from 90 genera are entomopathogens (Khachatourians and Sohail, 2008). They have many desirable traits that favour their development as bio-control agents (Lacey *et al.*, 2015). Entomopathogenic fungi occur naturally, are non-toxic to the environment (Copping, 2004), pose minimal risk to beneficial organisms such as bees (Lacey *et al.*, 2015) and problems with resistance are less likely to occur because of multiple modes of action (Zimmerman, 2007a). They have received considerable interest because they can serve as a component within an integrated pest management system. Entomopathogenic fungi have a unique mode of

infection; unlike other entomopathogens they do not need to be ingested but they invade the host directly through the cuticle (Sinha *et al.*, 2016).

Eilenberg *et al.* (2001), Lacey *et al.* (2001) and Shah and Pell (2003), listed four ways by which epf can be employed in IPM: classical biological control, inoculative biocontrol, inundative biocontrol and conservation biological control.

Entomophthorales have been used primarily for classical and conservations biological control and Hypocreales for augmentation biological control that includes inoculative and inundative control (Milner, 1997; Shah and Pell, 2003). Some species have already been commercialised for control of various species of thrips and aphids (Alves *et al.*, 2002). There are more than 100 commercial available products based on entomopathogenic fungi (Jaronski, 2014). However, the output of registered products arising from considerable research focus on microbial pesticides by African-based national and international research has been minimal (Grzywacz *et al.*, 2009). To date, various species/strains of entomopathogenic fungi such as *Lecanicillium* sp. (Kim, 2004; Jung *et al.*, 2006), *B. bassiana* (Quesada-Moraga *et al.*, 2006), *M. anisopliae* (Shi and Feng, 2004; Wright *et al.*, 2004) and *Nomuraea rileyi* (Farlow) Samson (Devi *et al.*, 2007) have been used for the management of aphids and other pests. Fungi in the Entomophthoromycota are known to cause field-wide epizootics among certain aphid species, for example: pea aphids, cereal aphids and cabbage aphids are all highly susceptible to *P. neoaphidis*, while cotton aphids are frequently infected by *N. fresenii* (Milner, 1997).

Mitosporic fungi such as *B. bassiana*, *Lecanicillium lecanii* (= *Verticillium lecanii*), *Isaria fumosorosea* (= *Paecilomyces fumosoroseus*) and *M. anisopliae* have been studied as common natural enemies of aphids and other agricultural pests (Milner, 1997; Shi and Feng, 2004; Roberts and St. Leger, 2004; Wang *et al.*, 2004; Li and Sheng, 2007). These species have also been commercialised, for example, about 37.2%, 36.4%, 5.8% and 4.1% of mycoinsecticides are based on *B. bassiana*, *M. anisopliae*, *Isaria fumosorosea* and *B. brongniartii*, respectively and the remaining percentage (22.3%) is from other species with negligible contribution (Jaronski, 2014). *Beauveria bassiana* and *V. lecanii* have a dual mode of actions, capable of parasitizing both insect pests and plant pathogens (Goettel *et al.*, 2008; Ownley *et al.*, 2010).

Hyphomycetes are inexpensive to mass produce, easy to store and efficient over an extensive range of temperatures and humidities (Lacey *et al.*, 2001; Akmal *et al.*, 2013) and have the potential to suppressing cereal aphids of economic importance (Hatting *et al.*, 2004; Murerwa *et al.*, 2014a, 2014b).

(a) *Beauveria bassiana*

Beauveria bassiana (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) has a broad host range of about 700 insect species (Feng *et al.*, 1994), grows in soil as a saprophyte (Chong-Rodriguez *et al.*, 2011) and as an endophyte in plants (Zimmerman, 2007a; Boomsma *et al.*, 2104). This insect pathogen is used for management of several crop insect pests (Amutha *et al.*, 2010, Sedighi *et al.*, 2012), including Coleoptera, Isoptera (Tamuli and Gurusubramanium, 2011), Diptera, Hemiptera, Lepidoptera, Orthoptera and Thysanoptera (Hajek, 2004; Wraight *et al.*, 2010). The white muscardine caused by *B. bassiana* was the first disease in animals to be caused by a fungus (Tanada and Kaya, 1993).

Beauveria bassiana possesses many strains that exhibit significant variation in pathogenicity, virulence and host range (Khetan, 2001). Effectiveness of *B. bassiana* SGBB8601 compared with other entomopathogenic fungi was demonstrated by Feng *et al.*, (1990a), when it killed more cereal aphid species than *L. lecanii* DNVL8701. However, *M. persicae* and *A. gossypii* were more susceptible to *L. lecanii* 41185 infections than of *B. bassiana* J57 at 25 °C and 75% relative humidity (Vu *et al.*, 2007). Feng *et al.* (2004) reported that *P. fumosoroseus* Pfr116 tended to be more effective than *B. bassiana* SG8702 against *Trialeurodes vaporariorum* but there was no significant difference in whitefly control. On contrary, *M. anisopliae* has not been reported to naturally infect cereal aphids.

(b) *Metarhizium anisopliae*

Metarhizium anisopliae Petch (Hypocreales: Clavicipitaceae), known as the 'green muscardine' fungus (Zimmerman, 1993) has been isolated from more than 200 insect species but has a restricted host range compared to *B. bassiana* (Zimmerman, 2007b). It is also a promising candidate for use in an IPM system (Pachamutu *et al.*, 1999; Schneider *et al.*, 2013). Various strains of *M. anisopliae* have been used to control a number of agricultural pests and domestic pests see (Table 1.1). However,

Zimmerman (1993) stressed that *M. anisopliae* strains differ considerably in their host range and therefore bioassays are needed to select the most virulent strain against a specific target insect, such as RWA in this instance.

Table 1.1: List of agricultural and domestic pests where various *Metarhizium anisopliae* Isolates were successfully used as a biological control agent.

Insect pest	Reference
Lettuce root aphid, <i>Pemphigus bursarius</i> Linnaeus	Milner and Soper, 1981, Chandler, 1997
<i>R. padi</i> and <i>M. dirhodum</i>	Murerwa <i>et al.</i> , 2014a,b
Taro beetles, <i>Papuana uninodis</i>	Faithpraise <i>et al.</i> , 2014
June beetles, <i>Polyphylla fullo</i> Linnaeus	Erler and Ates, 2015
Exotic wireworms <i>Agriotes lineatus</i> Linnaeus and <i>Agriotes obscurus</i> Linnaeus	Ericson <i>et al.</i> , 2007
Western flower thrip, <i>Frankliniella occidentalis</i> Pergande	Azaizeh <i>et al.</i> , 2002; Maniania <i>et al.</i> , 2003; Ansari <i>et al.</i> , 2007
<i>Reticulitermes flavipes</i> Kollar	Wright <i>et al.</i> , 2005
Plant hopper, <i>Nilaparvata lugens</i> Stal	Li <i>et al.</i> , 2012
Yellow fever mosquito, <i>Aedes aegypti</i> Linnaeus	Paula <i>et al.</i> , 2011
German cockroach, <i>Blattella germanica</i> Linnaeus	Pachamuthu <i>et al.</i> , 1999; 2000
<i>M. persicae</i>, <i>M. dirhodum</i>, <i>S. avenae</i>, <i>R. padi</i>, black bean aphid, <i>Aphis fabae</i> Scopoli and pea aphid, <i>Acyrythosiphon pisum</i> Harris	Hesketh <i>et al.</i> , 2008
<i>M. persicae</i>	Shan and Feng, 2010

1.4.1 Infection process

For an entomopathogenic fungus to be successful in the environment, it should be able to infect and suppress the host (Shah and Pell, 2003). Access to the host is through the cuticle and may comprise complex biochemical interactions between the host and the fungus before germination, penetration, growth and reproduction of the fungus can occur (Lacey *et al.*, 2001). Pathogenesis involves infection structures that are presumed to have evolved as a mechanism by which the pathogen incapacitates host barriers and produce toxic metabolites (Hajek and St. Leger, 1994). Infection process requires adhesion aiders, penetration effectors, enzymes and toxins (Samson *et al.*, 1988; Mustafa and Kaur, 2008). Adhesion of a conidium to the cuticle is the first step of pathogenesis (Hajek and St. Leger, 1994; Revathi *et al.*, 2011). But infection can be terminated if there is low humidity (Meyling and Pell, 2006) or if the fungus is unable to utilize nutrients on the cuticular surface, and if factors required for susceptible host recognition are absent (St. Leger, 1991).

Germination is marked by development of germ tubes or infection pegs from appressoria (Knudsen and Schotzko, 1999; Mustafa and Kaur, 2008). The inability of fungi to penetrate the cuticle has been attributed to the presence of inhibitory compounds such as phenols and lipids that are on the cuticular surface (Hajek and St. Leger, 1994). Cuticle penetration is aided by various enzymes that cause hydrolysis, followed by multiplication in the haemocoel (Boucias and Pendland, 1991; Fournier and Brodeur, 2000; Khan *et al.*, 2012; Sinha *et al.*, 2016). Once penetration has occurred, the epf reaches the haemolymph where it forms hyphal bodies (Mustafa and Kaur, 2008). Li and Holdom (1994) and Ambethgar (2009) emphasized that germination, mycelial growth and sporulation are three important events during infection and disease transmission. Vu *et al.*, (2007) suggested that these should be considered when selecting cultures for mycopesticide development. The host may be killed by nutrient exhaustion, toxicosis or mechanical damage caused by fungal growth (Hajek and St. Leger, 1994; Khan *et al.*, 2012). The relative importance of these mechanisms in fungal-host interaction might vary, depending on the epf isolate and the insect pest. *Beauveria bassiana* and *M. anisopliae* produce various toxic compounds or secondary metabolites during the infection process (Gillespie and Claydon, 1989; Wang *et al.*, 2004; Revathi *et al.*, 2011; Vega *et al.*, 2012). However, the role of these toxins in pathogenesis is not well understood. The amount of cyclic depsipeptides called

destruxins produced by *M. anisopliae* correlate with toxicity and the level of virulence of the pathogen against some insects (Kershaw *et al.*, 1999). On the contrary, *B. bassiana* produces the cyclic depsipeptides beauvericin, bassianolide, oosporein (Hajek and St. Leger, 1994), enniatins and bassianolide (Vey *et al.*, 2001). Certain limitations have caused the commercialisation of these agents to be slow. Faria and Wraight (2001) discussed a few factors that limit commercial development of fungi: potentially negative interactions with commonly used fungicides; limited shelf-life; and dependence on favourable environmental conditions. The slow killing effect seem to be a major hindrance (Lacey *et al.*, 2015), but Bateman and Alves (2000) believe certain environmental conditions are more prohibitive.

1.4.2 Entomopathogenic fungi infecting cereal aphids

Cereal aphids are susceptible to fungi in both the Entomophthoromycota and Ascomycota, but the entomophthoralean fungi are considered the major fungal pathogens of aphids (Humber, 1991; Roy *et al.*, 2006). Host death following Hyphomycetes infections is normally associated with toxin production which overcomes host defence responses, whereas host death by Entomophthorales tends to occur due to tissue colonization with little or no use of toxins (Shah and Pell, 2003). Entomophthorales have been found to cause natural epizootics in different cereal aphid species. For example, in a survey that was conducted between 2006 and 2007 in Egypt, *R. padi* and *S. graminum* were infected with six species of entomopathogenic fungi namely: *P. neoaphidis*, *N. fresenii*, *Entomophthora planchoniana*, *Zoopthora radicans*, *C. coronatus* and *L. lecanii* (Moubasher *et al.*, 2010). But *P. neoaphidis* and *N. fresenii* were the most dominant with 42% and 24% infected cadavers from all the sampled aphids, respectively. Moreover, *C. obscurus* and *P. neoaphidis* were the only entomopathogenic fungi that were found parasitizing *D. noxia* in southern Alberta and Saskatchewan in 1989 (Olfert *et al.*, 2002).

A similar study conducted in southwestern Idaho reported that *P. neoaphidis* was the most prevalent species occurring annually in *D. noxia* populations (Feng *et al.*, 1990b). A survey conducted by Wraight *et al.* (1993) reported almost the same result where the three dominant species recorded in irrigated areas were *Entomophthora chromaphidis*, *P. neoaphidis* and *C. obscurus* at 13%, 44% and 20%, respectively. However, very low levels of entomophthoralean fungal infection were observed in dryland areas.

In South Africa, a survey of fungal entomopathogens infecting cereal aphids was conducted from mid-90s to 1998 and six Entomophthorales and two Hyphomycetes were collected. Collected aphids were infected with *P. neoaphidis*, *C. thromboides*, *C. obscurus*, *Entomophthora planchoniana*, *Neozygites fresenii* and the two hyphomycetous fungi, *B. bassiana* and *L. lecanii* (Hating *et al.*, 1999). Russian wheat aphid was affected by almost all entomopathogens collected in the survey except *C. coronatus* and *E. planchoniana* (Hatting *et al.*, 1999). *Beauveria bassiana* and *L. lecanii* only occurred on *D. noxia* (Hatting *et al.*, 1999). *Lecanicillium lecanii* is considered the most important ascomycete parasitizing aphids (Milner, 1997); and has a broad host range, but is distributed mainly in tropical regions (Moubasher *et al.*, 2010). However, this fungus rarely affects aphids under conditions (Feng *et al.*, 1990b; Hatting *et al.*, 1999) and requires high levels of humidity. That will hinder its performance in the eastern Free State because of the climatic conditions that prevail in the region.

Despite the fact that most species of Entomophthorales, such as *P. neoaphidis*, are responsible for remarkable epizootics in *D. noxia* populations as reported by Feng *et al.*, (1990b) and Wraight *et al.*, (1993). Epizootics often occur late in the growing season after the aphids have reached the economic threshold (Mesquita *et al.*, 1996). As a result, inundative biological control is the most suitable method of employing fungi for insect control (Mesquita *et al.*, 1996). However, Shah and Pell (2003) argued that the use of inundative mycoinsecticides may not be as sustainable as non-inundative but they are more likely to be successful. This is because, the production of entomophthoralean fungi on artificial media is expensive, and they need high relative humidity close to 100%, to germinate, sporulate and infect (Milner, 1997). The shelf-life of conidia is normally too short to enable large scale production (Mesquita *et al.*, 1996). On the contrary, several species of fungi in the Sordariomycetes are more suitable for development as mycoinsecticides since they are cheap to mass produce, form stable conidia and have a reasonably good shelf-life (Milner, 1997).

Several Hyphomycetes species such as *I. fumosorosea* have been used to successfully manage RWA population (Mesquita *et al.*, 1996) and a synergistic effect with between the parasitoid *Aphelinus asychis* and *I. fumosoroseus* against *D. noxia* has been observed (Mesquita and Lacey, 2001). Vandenberg, (1996) found that *P. fumosoroseus* ARSEF 4461 and Mycotech612 were more virulent than *B. bassiana*

ARSEF4100 and Mycotech726 strains. *Beauveria bassiana* required three times more conidia to kill 50% of a RWA population tested than *P. fumosoroseus*, and also the LT₅₀ values of *P. fumosoroseus* were less than that of *B. bassiana*.

Vandenberg *et al.* (2001) reported significant reductions in pest densities and percentage tiller infestation following applications of *B. bassiana* GHA to *D. noxia* populations. Russian wheat aphids that were treated with the fungus had overt mycosis ranging from 32-83% (Vandenberg *et al.*, 2001). However, significant reductions were only observed 14 days after treatment with *B. bassiana* GHA, underscoring the need to enhance epf efficacy by reducing period of infection to death. To date, no studies have been conducted in SA to test the pathogenicity of indigenous strains of *B. bassiana* against RWA. The only study conducted locally was with an exotic strain formulated in the product Mycotrol® ES produced by Mycotech Corp., Butte, Montana, USA (Hatting *et al.*, 2004).

1.5 Methods of enhancing efficacy of entomopathogenic fungi

Mycoinsecticides are used as an environmentally safe alternative to chemical pesticides for insect control (St. Leger *et al.*, 1996). Nevertheless, certain limitations such as slow killing effect seem to be a major hindrance in commercialisation of these insect regulators (Lacey *et al.*, 2015). Bateman and Alves (2000) believe certain environmental conditions are more prohibitive. A way of improving the efficacy of entomopathogenic fungi is to reduce their latent period of infection and to engineer the epf to survive in extreme conditions.

1.5.1 Combining entomopathogenic fungus with oil

Feng and Pu (2005) found that a mineral oil-based emulsion used to formulate *B. bassiana* enhanced fungal activity against brown plant hopper, *Nilaparvata lugens* Stål, and did not cause any significant impact on background mortality. These results are similar to those obtained by Brito *et al.* (2008) who found that vegetable oil increased efficacy of *B. bassiana* against adult guava weevil, *Conotrachelus psidii* Marshall by increasing mortality by 30.7% compared to *B. bassiana* in a Tween 80 suspension. However, the presence of a sublethal dose of imidacloprid increased the percentage weevil mortality even more, to 88.6%. Additionally, Bateman and Alves (2000) indicated that the use of oils has been shown to improve the efficacy of mycoinsecticides thus allowing fungal pathogens to remain viable under low humidity

conditions. However, delivery systems for mycoinsecticides using oil-based formulations need further research because large droplets are associated with the use of air-shear and hydraulic nozzles that may reduce the efficacy of bioinsecticides by causing pesticide drift, bouncing from the foliage and not landing on target sites (Bateman and Alves, 2000).

1.5.2 Manipulation of host behaviour

Another approach highlighted by Roditakis *et al.* (2000) is increasing secondary conidia pick-up from surrounding vegetation through manipulation of host behaviour. The study indicated that systemic application of one percent of recommended dose of imidacloprid dramatically increased *M. persicae* movement. This is another possible approach that researchers need to look at.

1.5.3 Genetic manipulation of entomopathogenic fungi

Adding insecticidal genes to the entomopathogenic fungus has a potential to improve the fungal virulence (St. Leger *et al.*, 1996; Lacey *et al.*, 2015). An example of a recombinant fungal pathogen with enhanced virulence was shown when additional copies of the gene encoding the regulated cuticle-degrading protease Pr1 were inserted into the genome of *M. anisopliae* and were over expressed (St. Leger *et al.*, 1996). The resultant strain had 25% mean reduced survival times (LT₅₀) towards tobacco hornworm, *Manduca sexta* Linnaeus as compared to parent wild-type strain (St. Leger *et al.*, 1996). The notable extent to which virulence can be increased was also shown by expressing a scorpion, *Androctonus australis* Linnaeus toxin (AaIT, which is one of the most toxic insect-selective peptide) into *M. anisopliae* strain ARSEF 549 (Wang and St Leger, 2007). The modified fungus achieved the same mortality rates in *M. sexta* at 22-fold lower spore doses than the wild type, and survival times at some doses were reduced by 40% (Wang and St Leger, 2007). Furthermore, Pava-Ripoll *et al.* (2008) demonstrated that recombinant strain of *M. anisopliae* (AaIT-Ma549) significantly increased mortality of coffee berry borer (CBB), *Hypothenemus hampei* Ferrari. In the same study, median lethal concentration was also reduced by 15.7-fold and also average survival time was reduced by 20.1% and that was for the very first time that an epf was found to kill CBB in less than 3 days. Nevertheless, modified strain produced significantly fewer spores on treated cadavers than parental strain. Besides, Lu *et al.* (2008), showed that coexpression of Pr1 and AaIT failed to achieve any synergistic effect in *B. bassiana* since the expressed AaIT was digested

by Pr1 produced in the hemolymph. Studies discussed above clearly indicated that this field of research needs to be thoroughly investigated.

Genetic engineering strategies still require information on the roles and consequences of suitable genes that can be used to increase virulence leading to enhanced exploitation of the resources present in insect pathogenic fungi (St. Leger and Wang, 2010). Efficacy of entomopathogenic fungus can also be enhanced by combining insect pathogenic fungi with sub-lethal doses of insecticides. It is actually the two materials assisting each other, chemical act as a stress inducer and make the insect pest to be more susceptible to fungal infection (Benz, 1971; Ambethgar, 2009). Stressed insects are more susceptible to epf infection (Malekan *et al.*, 2012). Seyed-Talebi *et al.* (2014) proposed that application of microbiological control agents with minimum concentrations of acaricides is a very suitable approach in IPM.

1.5.4 Integration of entomopathogenic fungus with sub-lethal doses of insecticides

Among other commonly described drawbacks of using epf as a solo entity, is an extremely high inoculum rates required to cause statistically significant reductions in the pest population (Boucias *et al.*, 1996). Integrating a sub-lethal dose of imidacloprid with *M. anisopliae* and *B. bassiana* has proved to reduce the LT₅₀ in the dengue vector, yellow fever mosquito, *A. aegypti* (Paula *et al.*, 2011) and *N. lugens* (Feng and Pu, 2005). However, survival and establishment of entomopathogenic fungi can be negatively affected by chemicals used to control pests in the field (Mohammed *et al.*, 1987; Alizadeh *et al.*, 2007). Therefore, careful evaluation of the effects of chemical pesticides on the entomogeneous fungi is needed for their compatible use in IPM (Gardner *et al.*, 1979). Germination is regarded as the important criteria to evaluate the compatibility of pesticides with entomopathogenic fungi *in vitro* (Anderson and Roberts, 1983; Neves *et al.*, 2001). Even though other insecticides inhibit the fungal growth parameters such as germination and vegetative growth at recommended dose. Low insecticide concentrations are less likely to affect fungal sporulation (Anderson *et al.*, 1989). In addition, the beginning of the onset of an epizootic is associated with the capacity of germination of epf on the host (Anderson and Roberts, 1983). Incompatible insecticides may also inhibit the development and reproduction of these pathogens (Schumacher and Poehling, 2012).

1.5.5 Enhancing efficacy of entomopathogenic fungi through combination with botanical insecticides, pyrethrum

Bioinsecticides of botanical origin have gained a great deal of importance together with organic agriculture (Gokturk and Mihli, 2016), and they are promising alternative for pest management (Ribeiro *et al.*, 2012). Most of published research on compatibility of *B. bassiana* with botanical insecticides is on neem oil (*Azadirachta indica* A. Juss) and on other agricultural crops (see Depieri *et al.*, 2005; Ambethgar, *et al.*, 2009; Islam *et al.*, 2009; Sahayaraj *et al.*, 2011; Islam and Omar, 2012; Ribeiro *et al.*, 2012; Togbe *et al.*, 2015). No studies have been reported on combination of pyrethrum with *B. bassiana* against Russian wheat aphids in South Africa. This will be the first study to tackle this phenomenon. However, commercial formulation of *B. bassiana* (GHA) with pyrethrins indicated that there is synergism between *B. bassiana* and pyrethrins. Since, interaction is strain dependent, it's important to know if South African formulation Eco-Bb will show synergism with botanical based pyrethrum products.

Published research is based on comparing these control strategies as single entities. When toxicity of Naturalis® (*B. bassiana*) and Biopiren® plus (pyrethrins) was tested on different mite species found on vegetable crops in Italy, the effect was species dependent (Castagnolli *et al.*, 2005). *Beauveria bassiana* was not toxic to *Tetranychus urticae* Koch and *Tydeus californicus* Banks but induced high mortality in the progeny of treated females of *Neoseiulus californicus* McGregor. However, pyrethrins increased *T. urticae* fecundity but decreased *N. californicus* fecundity (Castagnolli *et al.*, 2005). It will be really interesting to find out how will *D. noxia* react to these insecticides. Pyrethrum (Spruzit® Neu, 600ml/Litre) was found to be effective on adult of *Ricania stimulans* Walker causing 96% mortality (Gokturk and Mihli, 2016). Lo *et al.* (1999) found pyrethrum (Night-time) at 0.5% and 0.25% mixed with mineral to be more effective on *M. persicae* as compared to *B. bassiana* (BotaniGard ES). Tested pyrethrum concentrations reduced aphid population from more than 20 aphids per shoot to less than 5 aphids per shoot. Shiberu *et al.* (2013) also controlled onion thrips using natural pyrethrum var. E-185 and *B. bassiana* PPRC-6 as a single entity in Ethiopia. The study showed that pyrethrum caused 60.79% on the 3rd day mortality and declined after 3 days whereas *B. bassiana* 46.1% at days and increased thereafter to 60.67% on the 7th day. From these findings, it is apparent that pyrethrum and Eco-Bb® might assist one another such that Pyrethrum will be toxic at early stages when *B.*

bassiana is still undergoing infection process. By the time pyrethrum is not available on the environment after day 3 that is when Eco-Bb® should kill the remaining population.

1.6 Conclusions and recommendations

Insecticide resistance caused by excessive usage of chemical insecticides is the major driving force in seeking alternatives for insect control. Entomopathogenic fungi combined with sub-lethal doses of chemical or botanical insecticides is one of the promising strategy that needs to be well researched and applied in crop protection. Combining entomopathogenic fungi with sub-lethal dose/s of insecticides could reduce amount of insecticides currently used in South Africa as a whole, since the country is one of the four largest importers of pesticides in Sub-Saharan Africa. The same practice could reduce level of environmental pollution, cases of insecticide resistance and also amount of money spent by farmers on pesticides.

Enhancing efficacy of entomopathogenic fungi should also focus on combination of epf with other control agents for instance entomopathogenic bacteria such as *Bacillus thuringiensis* or entomopathogenic nematodes.

Lastly, such combinations have an ability to reduce average time to kill as epf are normally disregarded because of extended Lt_{50} as opposed to chemicals.

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Chapter 2

Pathogenicity of *Beauveria Bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin isolates against Russian wheat aphid, *Diuraphis noxia* (Hemiptera: Aphididae)

2.1 Abstract

The Russian wheat aphid was first detected in South Africa in 1978. It originated in Central Asia and then spread to East Asia, the Middle East, Africa, North America, South America, and Central Europe. Current management of RWA relies mainly on host plant resistance, with the first resistant cultivar, Tugela-DN, released in 1992 by ARC. Genetic resistance is supported by natural enemies, with periodic applications of insecticides if needed, especially against new RWA biotypes that cause damage to previously resistant cultivars. The virulence of three *Beauveria bassiana* and three *Metarhizium anisopliae* strains were evaluated against *D. noxia* biotype RWASA1. Adult apterae were inoculated by spraying 5ml of a 5×10^7 conidia ml⁻¹ suspension onto the aphids inside a Burgerjon spray tower, resulting in a deposition of ca. 1000 conidia per mm². Inoculated aphids were transferred to wheat seedlings (Cultivar 'Tugela') and kept in a glasshouse at 22.5°C, and a 14:10h (day: night) photoperiod. All six fungal isolates were found to be pathogenic to *D. noxia* (lowest mortality = 31%), but the level of their virulence varied between species and strains within species. Abbott-corrected percentage mortalities differed significantly ($p < 0.0001$). The most virulent species was *B. bassiana*, with Abbott-corrected mortalities ranging from 51.5 – 61.2% and 30.9 – 36.8% for *M. anisopliae*. Percentage overt mycosis caused by the most virulent strain of *B. bassiana* (SGI921) was 78.9%, as opposed to 60.3% by the most virulent strain of *M. anisopliae* (VOPI5). Mean time to mortality ranged from 4.5 – 5.4 days among the different isolates. *B. bassiana* Isolate SGI921, was the most pathogenic strain, and it could be considered for further development as a biocontrol agent against RWA.

2.2 Introduction

The Russian wheat aphid (RWA), *Diuraphis noxia* (Morvilko) (Homoptera: Aphididae), is the most damaging wheat (*Triticum aestivum* L.) pest in the world (Pathak *et al.*, 2007). This aphid became a serious pest on wheat in South Africa in the late 1970's

(Walters *et al.*, 1980). Du Toit and Walters (1984) stated that severe infestation by RWA may result in yield losses of up to 90% in susceptible South African wheat varieties. Effective control of RWA has been a significant challenge facing wheat producers in regions where this pest occurs (Tolmay, 2006). There are also several cases of insecticide resistance that have been reported within hemipterans (Shufran *et al.*, 1997; Foster *et al.*, 2000; Houndete *et al.*, 2010; Silva *et al.*, 2012). Moreover, the insecticides registered for the control of RWA are mostly broad spectrum systemic organophosphates (Nel *et al.*, 2002) which have been found to kill natural enemies that attack RWA (Prinsloo, 2006). In many countries the use of most organophosphates have been discontinued due to environmental and safety concerns, thus leaving few chemical control options available to producers (Tolmay, 2006). This creates a need to find alternative measures to control RWA.

The use of resistant varieties is considered to be a safe and economic strategy to protect wheat from RWA (Pathak *et al.*, 2007; El Bouhssini *et al.*, 2011). However, Belay and Stauffer (2007) indicated that the durability of insect resistance can be threatened by the development of new biotypes of the pest. Currently, four biotypes of RWA are known in South Africa (Jankielsohn, 2014). Worldwide, development of virulent RWA biotypes will continue to pose a serious threat in wheat growing regions (El Bouhssini *et al.*, 2011). Inclusion of entomopathogenic fungi (epf) in an integrated pest management approach (Akmal *et al.*, 2013) against the RWA has been proposed, given their broad host range (Khan *et al.*, 2012), low to non-toxicity to mammals and non-target organisms (Zimmerman, 2007a; Zimmerman, 2007b) and residue-free status (Copping, 2004). Moreover, microbial control agents have a complex mode of action. Therefore, it is not easy for a pest to develop resistance against them (Khan *et al.*, 2012). Furthermore, they have specialized adaptations to infect insects such as overcoming host immune systems and producing cuticle degrading enzymes (Ortiz-Urquiza and Keyhani, 2013).

Hypocrealean fungi can be mass produced and have a reasonable shelf-life (Akmal *et al.*, 2013). *Beauveria bassiana* and *Metarhizium anisopliae* have a wide host range and can be easily isolated from insects, soil and a phylloplanes of vegetation (Meyling *et al.*, 2006; Freed *et al.*, 2011). Feng *et al.* (1990) noted the potential of using *B. bassiana* to manage *D. noxia* and five other cereal aphids. Other research with *B.*

bassiana it's potential to control cereal aphids, *Schizaphis graminum* and *Rhopalosiphum padi* (Akmal *et al.*, 2013). In the United States, significant reductions in RWA densities and percentage tiller infestation following application of *B. bassiana* were demonstrated by Vandenberg *et al.* (2001). The pathogenicity of *B. bassiana* and *M. anisopliae* has been tested against the cereal aphids, *Metopolophium dirhodum* and *R. padi*, in Kenya (Murerwa *et al.*, 2014a). In these experiments *Metarhizium anisopliae* isolates were more virulent against both aphid species. One isolate, ICIPE 51, was tested further to determine its effect against different nymphal instars and adults of *M. dirhodum* and *R. padi* and also the effects of fungal infection of fecundity and intrinsic rate of increase was investigated (Murerwa *et al.*, 2014b). Obtained results indicated that susceptibility of *R. padi* and *M. dirhodum* to fungal infection increases with aphid maturity and both species were more fecund at early adulthood stage.

In South Africa, the potential for controlling *D. noxia* using *B. bassiana* was identified by Hatting *et al.* (1999) when the pathogen was found to naturally infect the pest under field conditions. A follow up trial was conducted to manage RWA by combining *B. bassiana* and host plant resistance, which resulted in about 60% control (Hatting *et al.*, 2004). Improved levels of control may be possible by selecting more virulent strains of either *B. bassiana* or *M. anisopliae*, although last mentioned is not known to naturally infect RWA under field conditions in South Africa.

The objective of this research was to determine the pathogenicity of selected *B. bassiana* and *M. anisopliae* strains against Russian wheat aphid under controlled glasshouse conditions.

2.3 Material and methods

The methodology used in the bioassay was adapted from Hatting and Wraight (2007). However, minor changes were made to suit the current bioassay.

(A) Preliminary screening to confirm pathogenicity

The aim of the trial was to assess the pathogenicity of some fungal strains on RWA. Rearing of RWA, the number of aphids inoculated, preparation of fungal suspensions and concentration of fungal suspension were the same for the second virulence

bioassay. The only difference was the method of inoculation. In the first bioassay, the aphids were sprayed together with the plant substrate, allowing also for secondary-pick up of conidia during movement and feeding.

(B) Bioassay to determine the level of virulence among the selected fungal strains

2.3.1 Rearing and maintenance of the Russian wheat aphid

A clone colony of biotype RWASA1 was established by transferring one adult aphid to *T. aestivum*, cultivar Tugela. Nymphs produced by that single aphid were further reared until the adult stage (about 8 days old) and then transferred to fresh plants to maintain an uncontaminated colony. The colony was maintained in a glasshouse at $\pm 23^{\circ}\text{C}$ and a 14:10 h (day: night) photoperiod. Every 2 weeks infested leaves were transferred onto clean plants allowing the aphids to move from infested to uninfested plants. In preparation for the bioassay, adults from the colony were transferred to clean wheat plants and kept there for 24 h to allow nymph production (viviparous). These adults were then removed and the nymphs reared into same-age adults, for use in the assay. Wheat plants of Tugela cv. were grown in seedling trays and kept in the glasshouse under natural light conditions. Two weeks after planting, leaves were trimmed and soil surface was covered with white sand to make it easy to spot cadavers that fell from the plant after inoculation (Figure 2.2A). Adult aphids were collected with a fine camel hair brush from the plants and transferred to seedlings that will be used for bioassay.

2.3.2 Fungal isolates screened against RWA

The six fungal strains used in the study were *B. bassiana* (Rooi444, Rooi396 and SGI921) and *M. anisopliae* (Vopi5, Mal20 and Cadzw110) isolated from soil and other insect species (Table 2.1). *Beauveria bassiana* strains were cultured on SDA (Sabouraud dextrose agar) and *M. anisopliae* was grown on media containing 10g glucose, 5g peptone, 1g yeast and 7.5 water agar in 500 ml distilled water. All six fungal strains were passed through and re-isolated from *Galleria mellonella* Linnaeus larvae about 3 weeks before the bioassay was conducted in order to renew the virulence of the strains. Viability of the fungal isolates were tested ± 24 h before conducting the assay and all strains had germination $\geq 95\%$.

Table 2.1: Six fungal isolates of *Beauveria bassiana* and *Metarhizium anisopliae* used in the study, with corresponding origin and localities.

Fungal species	Isolate name	Accession number, PPRI*	Source	Locality	Year of collection
<i>B. bassiana</i>	Rooi 444	8132/7861	Soil from rooibos field	South Africa, Western Cape. S 32° 16' 798" E 18° 46' 571"	2003
<i>B. bassiana</i>	Rooi 396	7629/8096	Soil from rooibos field	South Africa, WC. S 31° 48' 529" E 18° 43' 126"	2003
<i>B. bassiana</i>	SGI 921	---	Dermaptera (earwig)	South Africa, Free State	2014
<i>M. anisopliae</i>	VOPI 5	8207	Disturbed soil	South Africa, Gauteng. S 25° 35' 000" E 28° 21' 000"	2003
<i>M. anisopliae</i>	Mal 20	--	Scarabaeidae (white grub)	Malawi	2011
<i>M. anisopliae</i>	Cadzw 110	---	Scarabaeidae (white grub)	Zimbabwe	2011

* Accessioned with the South African National Collection of Fungi, ARC-Plant Protection Research Institute, Pretoria.

2.3.3 Preparation of fungal suspensions

Conidial suspensions were prepared by suspending dry conidia into 0.01% Break-Thru® surfactant (Goldschmidt Chemical Corporation, Hopewell, VA, USA), homogenised with a hand-held blender and vortex mixer (at 2000 rpm for 1 minute Vibrofix VF1 electronic (IKA, Staufen, Germany), and adjusted with Nauber Haemocytometer to a concentration of ca. 5×10^7 conidia ml⁻¹ for all 6 fungal strains. Conidial suspensions were used within 2 hours after preparation.

2.3.4 Preparing inoculation platforms

Triangles with 30mm sides were cut from no.4 Whatman® filter paper (Merck NT Laboratory supplies (Pty) Ltd. Bloemfontein, South Africa) and submerged in about 20 ml of 0.01% Break-Thru® surfactant solution inside a 90 mm Petri dish (Figure 2.1B).

Thereafter, triangles were removed from the solution using forceps and placed on another disc of dry filter paper to absorb excess solution. The moist triangle was then transferred to a Petri dish containing a fine hypoallergenic talc (Johnson's Baby Powder, Johnson & Johnson®, East London, South Africa) which adhered to the under surface of the triangle. The forceps was used to gently press the triangle onto the powder to ensure adequate coverage (Figure 2.1B). The talc prevents the aphids from moving on to the underside of the triangle. The triangle was then removed from the Petri dish and positioned horizontally by securing the centre of one side with a 20mm alligator clip (AC/DC Dynamics, Driehoek, Germiston, Johannesburg, South Africa) glued to a 25mm steel nail at an angle of 90°. The nail was inserted into a ball of adhesive pretty (Prestic®, Permosel (Pty) Ltd., Beverly Roads, Montague gardens, Cape Town, South Africa) positioned on a pre-designated circle 18 cm diameter), drawn on the turntable of the spray tower, to reduce dose variation to possible deposition differences in the radial dimension.



Figure 2.1: Trimmed seedling and soil surface covered with fine sand (A); saturated triangles to be placed in Petri dish containing hypoallergenic talc (B); Russian wheat aphids positioned to ensure adequate spraying (C).

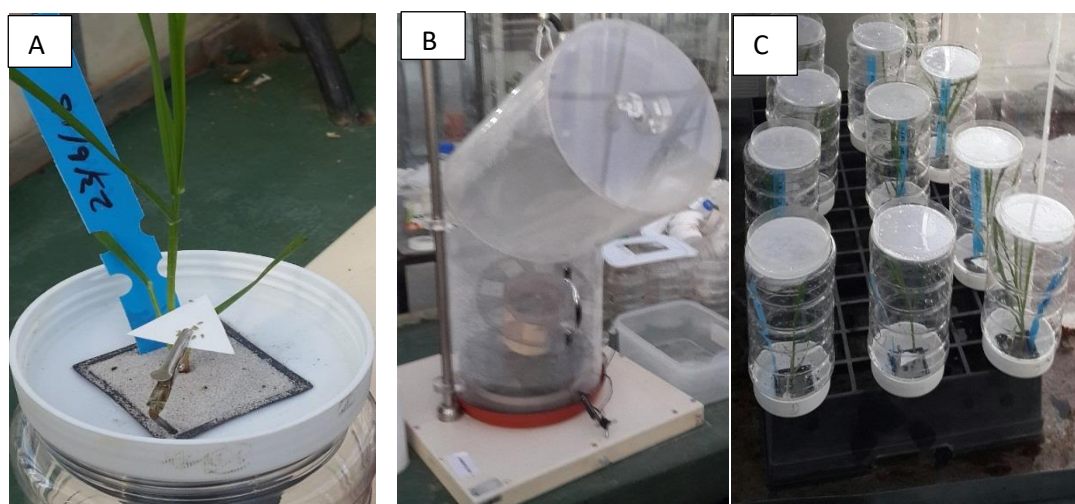


Figure 2.2: After spraying the triangle was positioned as to allow contact with the seedling (A); a Burgerjon spray tower used to inoculate the aphids during the bioassay (B); and (C) Clear inverted cages that were covered with moist filter paper and a 90mm Petri dish to enhance humidity in the cage

2.3.5 Inoculating the aphids

Adult aphids were agitated by exhaling warm air over the infested leaves thus to minimise control mortality. Eight hundred and forty (840) aphids were then collected from the rearing chambers by means of a fine camel hair brush and placed in 0.5ml Eppendorf® microtest tubes (Axygen Scientific, 33210 Central Ave., Union City, California, USA). In order to reduce aphid's mobility during spraying, they were kept in the cold room at 5°C for about 3 hours.

Five ml of each fungal suspension (see Section 2.3.2) and a control (0.01% Break-Thru® surfactant) were sprayed onto adult RWA placed on prepared triangles inside a Burgerjon spray tower (Figure 2.2A and B). This spray tower is a closed system calibrated with high precision. Each spray dose was quantified by counting the number of conidia deposited on a 65mm Petri dish containing 2% water agar.

Thereafter, inverted clear cages were covered with moistened filter paper and petri dishes to induce humidity for fungal germination (Figure 2.2C). Filter papers and Petri dishes were then removed after 24h. Aphids were kept inside clear ventilated inverted plastic cages for the duration of the bioassay. Treated aphids were inspected daily and cadavers were collected from Day One post inoculation up to 7 days post inoculation,

transferred to 65mm Petri dishes containing water agar and sealed with parafilm to allow fungal growth. Overt mycosis was confirmed by microscopic examination of cadavers.

Treatments consisted of a control (0.01% Break-Thru®) and fungal suspensions prepared from the 6 different isolates mentioned above (Table 2.1). Each treatment was replicated 4 times, with 10 aphids for each replicate, totalling 40 test insects. Three bioassays were conducted within a period of 2 months (May and June). The treatments were laid out in a Completely Randomised Design (CRD).

The measurements in this study were percentage mortality, percentage overt mycosis and mean time to mortality caused by each fungal strain.

2.3.6 Statistical analyses

The bioassays were conducted with a single dose of about 1000 conidia deposited per mm² for each fungal strain. Statistical packages used for analyses were SAS (1999) and XLSTAT (2011). The average number of conidia per mm² were analysed by ANOVA, using the General Linear Model (GLM) (SAS, 1999).

The data were normally distributed with acceptable homogeneous “trial” variances. Abbott’s formula was used to correct for control mortality (Abbott, 1925). The mean time of aphid mortality within 7 days post inoculation or spraying was calculated as follows: mortality was calculated for each of the four replicates. Thereafter, the number of aphids dying on a given day after inoculation was divided by the total mortality after 7 days. The obtained value was then multiplied by the corresponding day, and all the values from Day 1 to 7 were added to produce a weighted mean time of mortality for each treatment, prior to analysis. In all analyses, Fishers' unprotected t-test least significant difference was used to explain treatment effects when a significant *F* value ($P < 0.05$) was detected in the ANOVA (SAS, 1999).

2.4 Results

(A) Preliminary screening to confirm pathogenicity

The first bioassay showed that SGI921 was the best overall fungal isolate, although all strains were pathogenic against RWASA1. Abbott-corrected mortalities ranged from 31.2% to 81.9% (data not shown). The mean pooled mortality caused by *B. bassiana* (74±12%; overt 80±9.8%) was superior to that noted for *M. anisopliae* (36±5%; overt 58±3%).

(B) Virulence bioassay to determine level of virulence among the selected fungal strains

Germination percentages of all the tested strains were $\geq 95\%$. Application rate, percentage mortality, Abbott-corrected mortalities, percentage overt mycosis and mean time to mortality caused by selected *B. bassiana* and *M. anisopliae* strains are shown in Table 2.2.

A test for homogeneity of variance of application rate was done using Levene's and Bartlett's test. Levene's test had a p-value of 0.3548 and Bartlett's test had $\chi^2 = 3.7780$, P-value = 0.1512 and therefore the doses administered were considered equal. The data also followed a normal distribution pattern, where Kolmogorov-Smirnov for normality had a P. value = 0.1314, which is greater than 0.05. All the fungal strains tested differed for the parameters mentioned above.

Table 2.2: Mortality parameters in adult Russian wheat aphid, 7 days after treatment with a single dose of conidia of six isolates of entomopathogenic fungi.

Fungal strain	Number of aphids	Application rate (conidia/mm ²)	% Mortality	Abbott-corrected % mortality	% Overt mycosis	Mean time (d) to mortality
SGI921	120	989 ±34.1a	65.8±0.8a	61.2±0.7a	78.9±4.0a	4.5±0.2a
R396	120	1018±29.1a	59.1±3.7b	52.2±4.0b	71.2±1.0ab	4.6±0.2a
R444	120	979 ±17.5a	58.3±2.2b	51.5±0.1b	77.3±3.7a	4.6±0.1a
Vopi5	120	1076±31.1a	46.7±2.2c	36.8±0.7c	60.3±5.2bc	5.4±0.2b
Mal20	120	964 ±31.6a	42.5±1.5cd	34.7±3.2c	64.1±4.2bc	4.9±0.2ab
Cadzw110	120	975 ±23.8a	40.8±4.5d	30.9±2.1c	55.2±2.5c	4.8±0.2a
Control	120	-	14.1±4.4e	-	-	-

Means within a column followed by the same letter does not show significance difference at $\alpha = 0.05$

The mean mortality of the control was $14.2 \pm 4.4\%$; after the mortality data were subjected to Abbott-correction, the mortalities ranged from $30.9 \pm 2.1\%$ to $61.2 \pm 0.7\%$ for all the fungal strains (Table 2.2, Figure 2.3). A significant difference was noted between the Abbott-corrected mortalities ($P < 0.0001$, R-square = 0.94, CV = 8.37, LSD = 6.78). All the *B. bassiana* strains caused significantly higher mortalities ($51.5 \pm 0.1 - 61.2 \pm 0.7\%$) than the *M. anisopliae* strains, which were not significantly different from each other ($30.9 \pm 2.1 - 36.8 \pm 0.7\%$) (Figure 2.3).

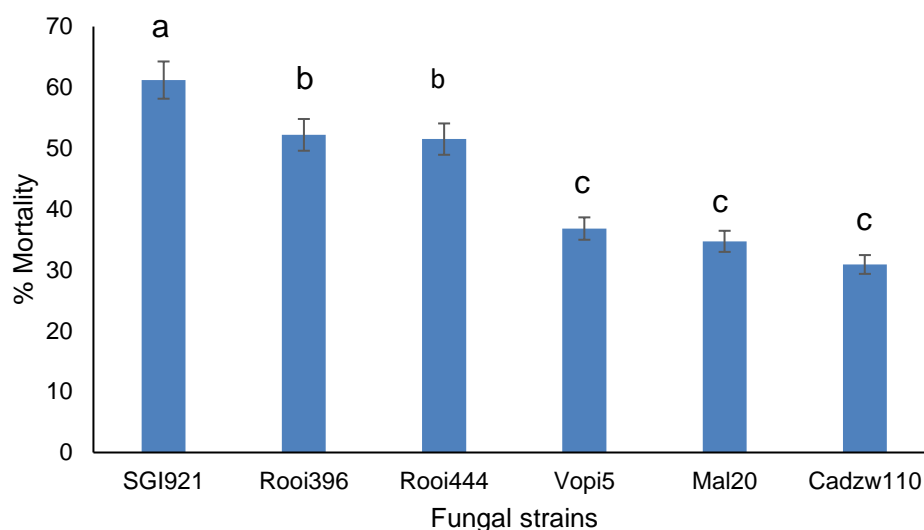


Figure 2.3: Abbott-corrected mortalities of Russian wheat aphids 7 days after treatment with different fungal strains.

Overt mycosis of collected cadavers differed significantly for the tested fungal strains (Figure 2.4). The General Linear Model showed a P value of 0.0089, LSD = 12.462, R-square = 0.74 and CV = 10.10). All *B. bassiana* strains showed higher overt mycosis when compared to the *M. anisopliae* strains.

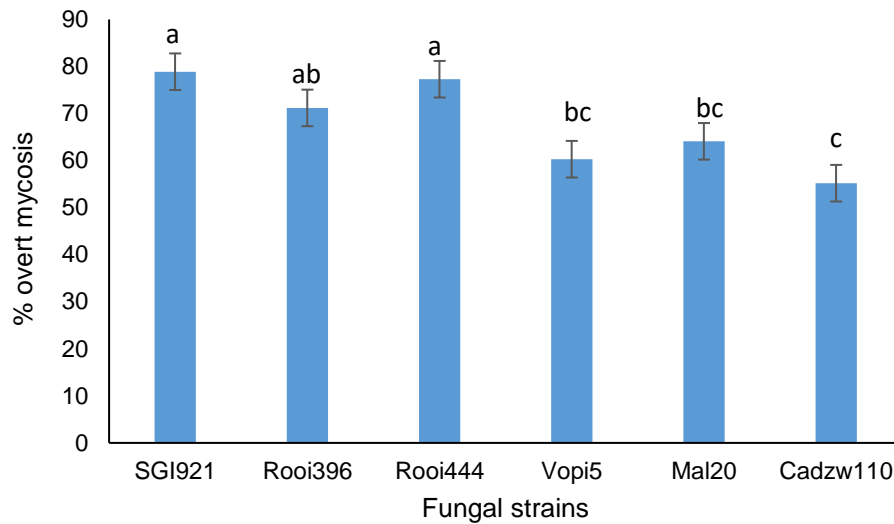


Figure 2.4: Percentage overt mycosis on Russian wheat aphid cadavers treated with two entomopathogenic fungal species.

There was a significant difference between treatments ($P < 0.0399$, $LSD = 0.5188$, $R\text{-square} = 0.69$) when the mean time to mortality was tested, measured in days post inoculation (Table 2.2, Figure 2.5). All three *B. bassiana* strains and Cadzw110 had significantly lower mean times to mortality ranging from 4.5 to 4.8 days). Notably, the mean time to mortality of all 6 entomopathogenic fungal strains was about 5 days.

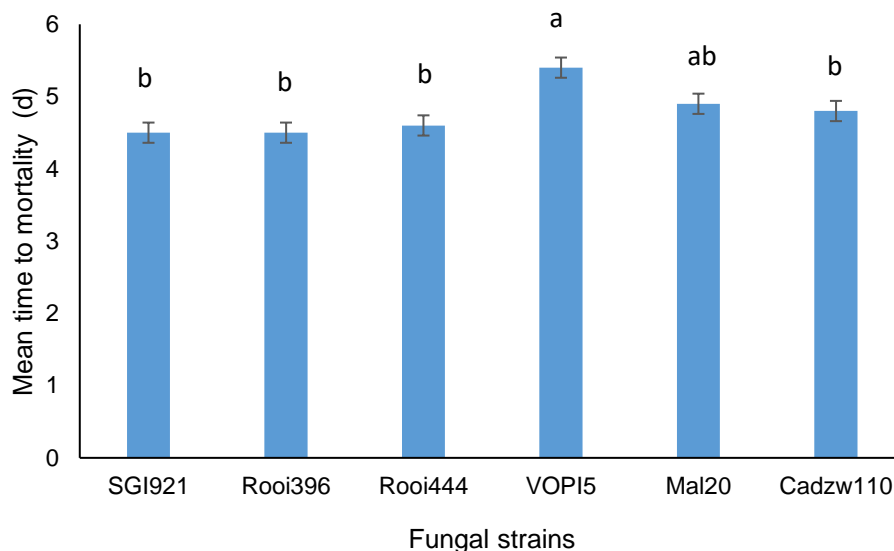


Figure 2.5: Mean time to mortality of RWA caused by three different *Beauveria bassiana* and *Metarhizium anisopliae* strains.

The best isolate (SGI 921) was selected on the basis of causing high percentage mortalities, percentage overt mycosis and significantly lower mean time to mortality.

2.5 Discussion and conclusions

The objective of the study was to determine virulence of selected *B. bassiana* and *M. anisopliae* strains against adults of RWA. All six fungal isolates were pathogenic to *D. noxia*, but the level of their virulence varied between species and isolates within species.

The preliminary trial, where the aphids were sprayed together with the plant substrate, resulted in increased percentage mortalities as compared to the virulence bioassay which was about 10% lower for all the tested fungal strains. These findings suggest that the aphids were exposed to unquantified secondary pick up when the aphids were sprayed together with the substrate. Secondary pick up of conidia was demonstrated with peach potato aphids, *Myzus persicae*, Sulzer when green pepper, *Capsicum annuum*, cv. California Wonder leaves were treated with *Lecanicillium lecanii* (Zimmerman) Viegas (Roditakis *et al.*, 2000) and also when western flower thrips, *Frankliniella occidentalis* Pergande were exposed to kidney bean, *Phaseolus vulgaris* Linnaeus leaf disks and garden impatiens, *Impatiens wallerana* Hook treated with *B. bassiana* (Ugine *et al.*, 2005; Ugine *et al.*, 2007).

Strains of both *B. bassiana* and *M. anisopliae* were effective against cowpea aphid, *Aphis craccivora* Koch at 1×10^7 conidia ml⁻¹, with percentage mortalities ranging from 53.1% - 86.4% for *B. bassiana* strains and 56.1% - 91.4% for the latter (Ekesi *et al.*, 2000). Liu *et al.* (2002) found both *B. bassiana* and *M. anisopliae* isolates to be competent bio-control agents of the tarnished plant bug, *Lygus Lineolaris* (Hemiptera: Miridae), causing percentage mortalities of 98% and 92%, respectively. The same outcomes were noted by Migiro *et al.* (2010) against pea leafminer, *Liriomyza huidobrensis* Blanchard (Diptera: Agromyzidae) and by Hussein *et al.* (2012) against greater wax moth, *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae) where the most virulent strains of both species caused 100% mortality five days after treatment. These results indicated that *B. bassiana* and *M. anisopliae* can have the same level of virulence. Similarly, no significant difference was noted between the two fungal species when isolates were tested against *G. mellonella* and mealworm, *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae) by Oreste *et al.* (2012).

As with our results, Vu *et al.* (2007) found that *B. bassiana* was more virulent against *M. persicae* than *M. anisopliae*, with LT₅₀ values of 4.75 and 5.66, respectively. Ibrahim *et al.* (2011) found that the LC₅₀ and LT₅₀ values for *B. bassiana* were lower against *M. persicae* and *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) than those of *M. anisopliae*. Faraji *et al.* (2013) also used the same concentration as our current study of 5×10^7 conidia ml⁻¹ against Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) and found that the strains of *B. bassiana* were more virulent than *M. anisopliae* strains.

Our results indicated that the three *B. bassiana* strains were more virulent than those of *M. anisopliae* against RWA, as indicated by percentage mortalities and percentage overt mycosis (Table 2.2, Figure 2.3 and 2.4). These findings are contrary to that of Murerwa *et al.* (2014a), where the same two fungal species were tested against *Rhopalosiphum padi* (Hemiptera: Aphididae) and *Metopolopodium dirhodum* (Hemiptera: Aphididae). In that study, *M. anisopliae* isolates were more virulent than *B. bassiana* against both aphid species and the most virulent *M. anisopliae* strain caused 91.7% mortality, whereas the most virulent *B. bassiana* isolate only caused 81% mean mortality.

Shapiro-Ilan *et al.* (2008) treated the black pecan aphid, *Melanocallis caryaefoliae* Davis (Hemiptera: Aphididae), with both *B. bassiana* (GHA strain) and *M. anisopliae* (F52 strain) at 1×10^7 and 1×10^8 spores ml⁻¹. The *Metarhizium anisopliae* strain caused a higher mortality (>90%) than the *B. bassiana* which caused more than 80% mortality, four days after treatment at the low concentration. However, at the high concentration, both isolates caused about 90% mortality. The Shapiro-Ilan *et al.* (2008) study was conducted in the laboratory in Petri dishes whereas the current study was conducted under glasshouse conditions.

Mburu *et al.* (2009) and Addisu *et al.* (2013), also found selected *M. anisopliae* isolates to be more virulent than *B. bassiana* isolates in terms of percentage mean mortalities, LT₅₀ values, and percentage mycoses at 1×10^7 conidia ml⁻¹ against the termite, *Macrotermes michaelseni* Sjöstedt (Isoptera: Termitidae, Macrotermitinae). The same results were obtained by Gindin *et al.* (2006) against the red palm weevil, *Rhynchophorus ferrugineus* Olivier (Coleoptera: Curculionidae) where mortality of treated larvae ranged from 0 to 20% for *B. bassiana* and 40 to 100% for *M. anisopliae*.

Metarhizium isolates killed the treated larvae quicker than the *B. bassiana* isolate, with LT₅₀ values of 3.5 days and 5.8 days, respectively. Loc *et al.* (2010) also found that *M. anisopliae* isolates were more virulent than isolates of *B. bassiana* against the citrus psylla, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) and the black citrus aphid, *Toxoptera* sp. (Homoptera: Aphididae). Vu *et al.* (2007) found *M. anisopliae* isolates to be more virulent against *Aphis gossypii* Glover (Homoptera: Aphididae) than isolates of *B. bassiana* with LT₅₀ values of 1.64 and 2.70. From all the results that have been discussed, it can be noted that virulence among the isolates of two entomopathogenic fungi, and that this varies depending on the target host. It is probable that different target host insects express differential levels of susceptibility against the specific isolates of both *B. bassiana* and *M. anisopliae*.

Percentage overt mycosis results confirmed that the greatest portion of mortality was due to fungal infection (Table 2.2, Figure 2.4), as opposed to poor handling and environmental conditions. A survey of fungal pathogens naturally infecting cereal aphids in South Africa indicated that *D. noxia* was more prone to infection by Entomophthorales than Hypocreales (Hatting *et al.*, 1999; 2000). This suggests that RWA may be more susceptible to Entomophthorales as compared to Hypocreales. However, it is difficult to grow and formulate entomophthoralean fungi as bio-insecticides because of their fastidious culture requirements and their intolerance to desiccation, complicating large scale production and application of these fungi (Mesquita *et al.*, 1996).

Other researchers have hypothesized that the most virulent fungal strains are best isolated from the test organisms (Liu *et al.*, 2002). However, our results are not in agreement with this theory because the RWA-derived Strain SGI192, was inconsistent in its ability to cause mortality and its germination percentage < 80% (data not shown). The most virulent fungal strain from this bioassay was isolated from an earwig (Dermaptera) and the second most virulent strain was isolated from soil. Lastly, *M. anisopliae* has not been recorded as being isolated from *D. noxia* in nature but was able to cause disease in this aphid even though the mortality percentage was less than 50%. Nevertheless, the species has the potential to control wheat aphids as shown by Murerwa *et al.* (2014a; 2014b). Therefore, screening of potential isolates should not be limited to those isolated from the target host. Feng and Johnson (1990) also stated that the host of origin and phylogenetic relationship between potential hosts is not a

guaranteed indicator of the probable virulence of a specific fungal isolate against a specific host. Lastly, Feng *et al.* (1990) found that *D. noxia* was more susceptible to a *B. bassiana* strain SGBB8601 isolated from *S. graminum* than strain *Lecanicillium lecanii* DNVL8701 isolated from *D. noxia*, confirming this point.

In conclusion, the study indicated that three selected *B. bassiana* isolates were more pathogenic against *Diuraphis noxia* than three *M. anisopliae* strains in glasshouse trials. The most pathogenic and virulent fungal strain was SGI921, both when the aphids were sprayed together with the substrate, and when aphids were sprayed and immediately transferred to un-inoculated wheat plants.

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Chapter 3

***In vitro* compatibility between selected systemic insecticides and *Beauveria bassiana* strain SGI921**

3.1 Abstract

One of the strategies to enhance the efficacy of entomopathogenic fungi (epf) is to integrate the microbial insecticide with sub-lethal doses of insecticides. Low doses can improve the control efficacy of epf if they do not affect the fungi. The *in vitro* compatibility of an epf, *Beauveria bassiana* Strain SGI921, with four chemical insecticides (active ingredients: chlorpyrifos, dimethoate, demeton-S-methyl and acetamiprid) was evaluated. To determine the impact of these insecticides on germination, radial vegetative growth and sporulation intensity, different concentrations ranging from 1% to 100% (of company-recommended application rate) were added to Sabouraud dextrose agar + 1% yeast extract (SDAY). Colony diameter (mm) was measured along a radial diameter in order to determine vegetative growth, while a 10mm disk was excised from a sporulating colony, mixed with surfactant, vortexed and then quantified using a Neuber Haemocytometer. All the tested insecticides reduced germination, radial vegetative growth and sporulation intensity of the selected strains to various degrees in a concentration-dependent manner. According to a biological index for the classification of the toxicological effect of chemical compounds on entomopathogenic fungi, all tested emulsifiable concentrates (chlorpyrifos, demeton-S-methyl and dimethoate) registered in South Africa for Russian wheat aphid control ranged from moderately toxic to toxic for concentrations of 50% and above. This implies that these insecticides have antagonistic effects and at these concentrations they should not be used in chemical-fungal combinations. Germination and sporulation was also reduced in relation to the control in the presence of Mospilan. Lastly, biological index showed that it was compatible with the selected strain and surprisingly, only the highest concentration seemed to have stimulated vegetative growth.

3.2 Introduction

Beauveria bassiana (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) is a major fungal entomopathogenic fungus (epf) that has been developed as a microbial insecticide for use against major arthropods in agriculture (de Faria and Wraight, 2007). It has a broad host range of about 700 insect species (Feng *et al.*, 1994; Usha *et al.*, 2014), grows in soil as a saprophyte (Chong-Rodriguez *et al.*, 2011) and grows endophytically in certain host plants (Ownley *et al.*, 2004; Quesada-Moraqa *et al.*, 2006; Akello *et al.*, 2007; Posada *et al.*, 2007; Vega *et al.*, 2008). It is used for management of several crop insect pests (Amutha *et al.*, 2010, Sedighi *et al.*, 2012), including Coleoptera, Isoptera (Tamuli and Gurusubramaniam, 2011), Diptera, Hemiptera, Lepidoptera, Orthoptera and Thysanoptera (Hajek, 2004; de Faria and Wraight, 2007; Wraight *et al.*, 2010). Moreover, *B. bassiana* has been found to naturally infect *Diuraphis noxia* (Mordvilko) in South Africa (Hatting *et al.*, 1999; Hatting *et al.*, 2000). Different *B. bassiana* strains have also been shown to infect other cereal aphids in Africa, such as *Metopolophium dirhodum* Walker and *Rhopalosiphum padi* Linnaeus (Murerwa *et al.*, 2014) and *D. noxia* (Hatting *et al.*, 2004).

Even though several species of hypocrealean fungi (e.g., *B. bassiana* and *Metarhizium anisopliae*) have been commercialised worldwide, their performance against r-strategists such as aphids (Dixon, 1985) is challenged by the high fecundity and short generation time associated with these insects (Dixon, 1987). One strategy to enhance the efficacy of entomopathogenic fungi and to accelerate insect mortality (Asi *et al.*, 2010) maybe to combine them with sublethal doses of insecticides (Schumacher and Poehling, 2012). Ambethgar (2009) discussed how the two approaches could assist each other, whereby the chemical stresses the insect making it more susceptible to fungal infection, and conversely, where the pathogen would make the pest sick enough to become less resistant to the chemical. Generally, sub-lethal doses of chemical insecticide used in combination with epf can increase the control efficiency of the fungus (Anderson *et al.*, 1989), provided that the selected sublethal dose of insecticide does not have a negative effect on the fungus.

Some insecticides can enhance the efficacy of epfs against insect pests, as shown by Quintela and McCoy (1998); Ye *et al.* (2005); and Purwar and Sachan (2006). On the other hand, other pesticides may have inhibitory effects on epfs (Asi *et al.*, 2010).

Interactions between insecticides and epfs can be classified as synergistic, antagonistic or neutral (Asi *et al.*, 2010). For this reason, it is important to understand the effect of each chemical insecticides on the germination, vegetative growth and sporulation intensity of selected microbial control agents.

The integration of microbial agents with chemical insecticides requires detailed compatibility studies (Amutha *et al.*, 2010). Data from such studies would enable farmers to schedule the application of combined microbial and chemical insecticides, in order to exploit the benefits of compatible combinations (Amutha *et al.*, 2010). Gardener *et al.* (1979) stated that integration of any fungal isolate with an insecticide requires a thorough understanding of the compatibility between the two agents; more especially the effects on spore germination, vegetative growth and sporulation (see also Pachamuthu *et al.*, 1999).

The noted adverse effects that chemical insecticides can have on entomopathogenic fungi include the inhibition of germination, vegetative growth, and reduced sporulation intensity. Therefore, chemical-fungal combination studies are warranted in order to eliminate the incompatible combinations that will result in significant inhibition of any of the fungal growth stages. The aim of this study was to evaluate the influence of selected insecticides on conidial germination, vegetative growth and sporulation of *B. bassiana* and to select a sub-lethal dose that would enhance the efficacy of *B. bassiana* *in vitro*. The chemicals tested with *B. bassiana* strain SGI921 were chlorpyrifos, dimethoate, demeton-s-methyl and acetamiprid.

3.3 Material and methods

The experiment was arranged in a completely randomized design (CRD) with four treatments, replicated three times.

3.3.1 Entomopathogenic fungi

Beauveria bassiana Strain SGI921 was selected, based on its superior performance in a prior the pathogenicity bioassay (see Chapter 2). The selected strain was initially isolated from an earwig (Dermaptera) collected at ARC-SGI, Bethlehem). The fungus was cultured on antibiotic-free Sabouraud Dextrose Agar + 1% Yeast extract (SDAY) in 65 mm diameter Petri-dishes and incubated for 2 weeks at $\pm 22^{\circ}\text{C}$. Harvested conidia

were used for the evaluation of conidial germination, radial vegetative growth and sporulation intensity.

3.3.2 Insecticides

Dursban™ (chlorpyrifos, 480 g/l, active ingredient), Dimethoate (dimethoate, 400g/l), Demeton (demeton-S-methyl, 250 g/l) were obtained as emulsifiable concentrates (EC) and Mospilan 20 SP (acetamiprid, 200g/kg) was purchased as a soluble powder formulation. Dursban, Dimethoate and Demeton are registered contact and systemic insecticides against Russian wheat aphid on wheat and belong to the organophosphate group of insecticides, whereas Mospilan (in the neonicotinoid group) is systemic and only registered against green and brown aphids (NDA, 2007; van Zyl, 2013). The insecticide dose was calculated for each insecticide given the registered field application rates. Obtained pesticides were used at seven different concentrations: 100, 75, 50, 25, 5, 2.5, 1% and 0% (control). Each concentration had 8 plates as replicates and the experiment was repeated three times within a period of 3 weeks. These concentrations were selected with the aim of identifying sublethal concentrations that would enhance fungal growth and general performance.

Table 3.1: Details of chemical insecticides tested for compatibility with *Beauveria bassiana* Strain SGI921.

Trade name	Active ingredient	Chemical name	Supplier	Recomme nded dose (g or ml/ hectare)	Recomme nded dose (g /ml) in 1Litre
Dursban™ EC	Chlorpyrifos 480g l ⁻¹	O,O-diethyl O-3,5,6- trichloropyridin-2-yl phosphorothioate	Dow Agrosciences Southern Africa (Pty) Ltd	750ml	3.75ml
Dimethoate EC	Dimethoate 400g l ⁻¹	O,O-dimethyl S-[2- (methylamino)-2- oxoethyl] dithiophosphate	Villa Crop Protection, South Africa	750ml	3.75ml
Demeton	Demeton-S- methyl 250 g l ⁻¹	O,O-Diethyl S-2- (ethylsulfanyl)ethyl phosphorothioate	Villa Crop Protection, South Africa	500ml	16.67ml
Mospilan	Acetamiprid 200g kg ⁻¹	N-[(6-chloro-3- pyridyl)methyl]-N'- cyano-N-methyl- acetamidine	Nulandis, South Africa	50g	0.167g

3.3.3 Conidial germination

Each insecticide of the above mentioned concentrations was added to $\pm 40^{\circ}\text{C}$ of 500ml SDA. The mixture was poured into 65 mm Petri dishes. After cooling, the culture media was inoculated with 1 ml of a conidial suspension containing 5×10^7 conidia ml^{-1} which was spread evenly over the plate (Alizadeh *et al.*, 2007). All treatments were kept in the incubator at $\pm 22^{\circ}\text{C}$ for 24 h. After incubation, percentage germination (4 plates) was computed by counting the number of germinated versus non-germinated spores out of 100 randomly selected conidia.

3.3.4 Vegetative growth

Ten (10) μl fungal suspension of 5×10^7 conidia ml^{-1} was inoculated aseptically at the centre of each insecticide-amended media and control plates in 65 mm Petri dishes. Control plates were SDA without insecticide. Eight Petri dishes were assigned to each treatment. Plates were then incubated at $\pm 22^{\circ}\text{C}$ for 7 days. Radial vegetative growth (colony diameter) was measured in mm and recorded from 2 days after inoculation (DAI) and 7 DAI in order to determine the change in growth from the first day post inoculation.

3.3.5 Sporulation intensity

Sporulation intensity of *B. bassiana* Strain SGI921, in the presence of various concentrations of insecticides, was assessed 9 days post inoculation (Rashid *et al.*, 2012). Five central colony disks of about 10 mm each were randomly excised from the sporulating colonies of each treatment with the sterile cork borer. Disks were then transferred to a McCartney bottle and suspended in 10 ml of a 0.01% Break-Thru[®] suspension, agitated and vortexed at 2000 rpm for 1 minute using a Vibrofix VF1 electronic vortex (IKA, Staufen, Germany) (Neves *et al.*, 2001). Thereafter, the conidial concentration on each treatment was quantified with Neauber Haemocytometer.

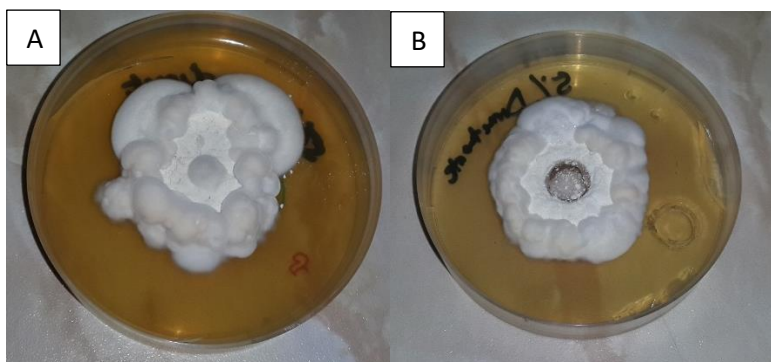


Figure 3.1: Sporulating *B. bassiana* colonies 9 days post inoculation: (A) about 19mm in diameter; (B) Centre of the colony was excised with ca. 10 mm sterile cork borer prior to mixing with a 0.01% Break-Thru® suspension.

3.3.6 Calculation of the compatibility of pesticides with *B. bassiana*

Inhibition levels were calculated using the formula adapted from Sahayaraj *et al.* (2011).

$$\text{Percentage inhibition} = [(\text{Control value} - \text{Treatment value}) / \text{Control value}] \times 100$$

A biological index for the classification of the toxicological effect of chemical compounds on entomopathogenic fungi growing *in vitro* on solid culture media was used (Alves *et al.*, 2007; Schumacher and Poehling, 2012). The index is based on the mean percentage germination (GR), sporulation (SP), and vegetative growth (VG) of the fungal colonies, calculated in relation to the Control (Schumacher and Poehling, 2012).

$$\text{Biological Index formula} = [47 \times \text{VG} + 43 \times \text{SP} + 10 \times \text{GR}] / 100$$

Ranking of BI values, BI > 66 (Compatible), $42 \leq \text{BI} \leq 66$ (moderately toxic) and BI < 42 (toxic).

3.3.7 Statistical analyses

A completely randomised experimental design was used in experiments. Each treatment was replicated 3 times. A combined analysis of variance (ANOVA) was performed over three different trials after testing for homogeneity of variances to test for the differences between strains. The standardized residuals were normally distributed (Shapiro-Wilks test) and the means were separated using Fisher's

unprotected t-test (least significant difference – LSD) at the 5% level of significance (Snedecor & Cochran, 1989).

3.4 Results

3.4.1 Conidial germination

The germination percentage of *B. bassiana* Strain SGI921 was influenced by the presence of the insecticides in a concentration-dependent manner for all three emulsifiable concentrates, followed by mospilan, as shown by percentage reduction over the Control (Table 3.2). There was a significant difference between insecticides ($P < 0.0001$) and also between insecticide concentrations ($P < 0.0001$). R-square was 0.999, CV = 1.95 and LSD = 3.539. Significant differences were found at $\geq 25\%$ concentrations of mospilan and chlorpyrifos, $\geq 5\%$ of demeton-S-methyl, and $\geq 1\%$ of dimethoate.

Demeton-S-methyl was found to be the most detrimental insecticide, completely inhibiting fungal growth at 25% and above, followed by dimethoate. The selected fungal strain tolerated seven tested concentrations of mospilan, which caused less than 5% reduction in growth relative to the Control (Table 3.2).

There was a strong linear correlation between germination percentage and insecticide concentration, as indicated by high R^2 values for all four insecticides. These ranged from 0.7139 to 0.8803 (Figure 3.2). A significantly negative correlation was shown by demeton-S-methyl with a slope of -18.649. As the concentration increased the germination percentage decreased. However, Mospilan line graph was almost parallel to the x-axis, which means germination percentage was almost constant for all the tested concentrations. A sharp decline in percentage germination was observed for 25% demeton-S-methyl, 50% dimethoate and 75% chlorpyrifos concentrations (Figure 3.2).

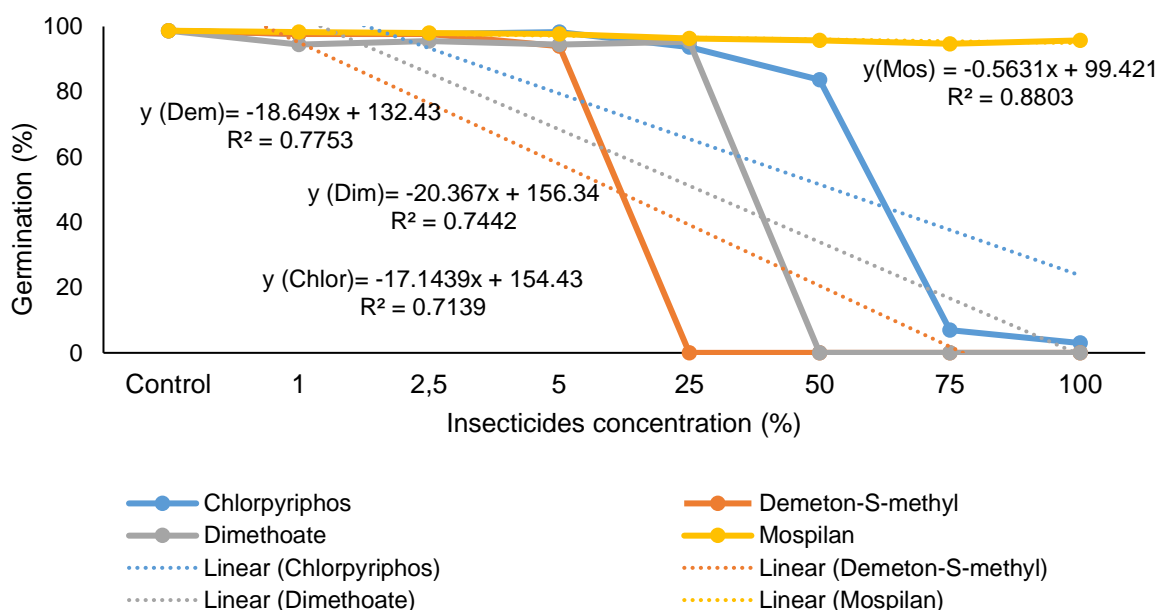


Figure 3.2: The effect of different insecticide concentrations on the germination of conidia of *B. bassiana* Strain SGI921.

3.4.2 Vegetative growth

At the 75% and 100% concentrations demeton-S-methyl, dimethoate and chlorpyrifos significantly I the radial vegetative growth, whereas mospilan appeared to enhance the vegetative growth of *B. bassiana* Strain SGI921 (Table 3.2). The interaction between the insecticides and concentrations was significant ($P < 0.0001$). Vegetative growth was increased at 75 and 100% mospilan concentrations, demeton-S-methyl at 2.5% and chlorpyrifos at 1%, 2.5% and 25% (Table 3.2). Insecticide concentrations of $\geq 5\%$ (demeton-s-methyl), $\geq 50\%$ (chlorpyrifos and dimethoate), and $\geq 75\%$ (mospilan) resulted in significantly increased radial vegetative growth of *B. bassiana* Strain SGI921 strain than control.

3.4.3 Sporulation intensity

Conidial sporulation was reduced by most of the tested concentrations ($P < 0.0001$) except for 2.5, 5, 50 and 75% mospilan (Table 3.2). The interaction between insecticide concentration and fungus was also significantly different ($P < 0.01$). Demeton-S-methyl and dimethoate were the most toxic insecticides, such that 100% reduction in sporulation intensity was observed for concentrations of 50% upwards. An inconsistent interaction between sporulation intensity and insecticide concentration was observed when the selected fungal strain was treated with mospilan.

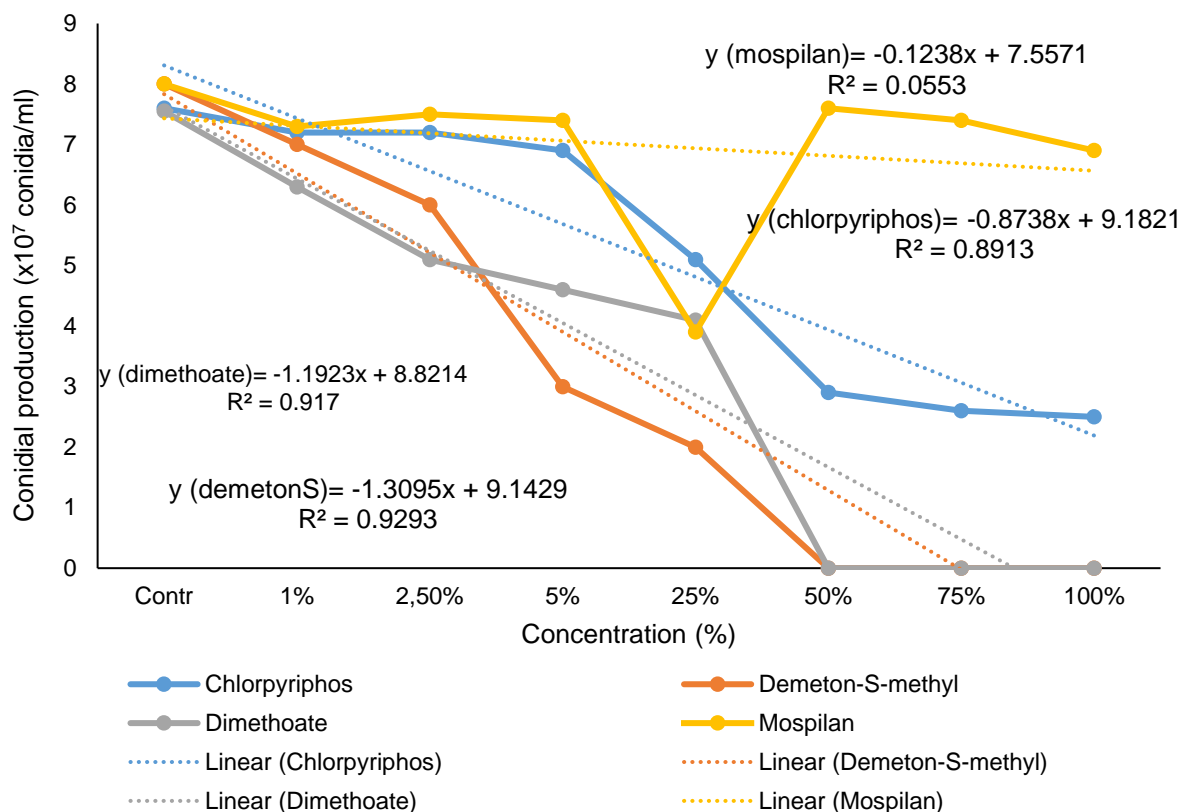


Figure 3.3: Sporulation intensity of *B. bassiana* Strain SGI921 in the presence of various concentrations of selected insecticides.

A strong negative correlation was observed between insecticide concentration and sporulation intensity for demeton-S-methyl ($R^2 = 0.929$), dimethoate ($R^2 = 0.917$) and chlorpyrifos ($R^2 = 0.891$) (Figure 3.2). However, a poor association was shown by mospilan, with a very weak coefficient of determination ($R^2 = 0.0553$) and slope (-0.1238), compared to chlorpyrifos (-0.8738) and demeton-S-methyl slopes (-1.3095).

3.4.4 Compatibility of *B. bassiana* with chemical insecticides

The biological index showed that all tested mospilan concentrations were compatible with *B. bassiana* Strain SGI921 with values closed to 100% (Table 3.3). Chlorpyrifos was also compatible at 0.5X recommended dose and below; 75% and 100% were moderately toxic with index values of 59.03% and 54.44%, respectively. Lastly, demeton-S-methyl was only compatible at the 5% concentration and below, 25% was moderately toxic (BI = 53.53) and any concentration $\geq 50\%$ was classified as toxic (BI = 0). Demeton-S-methyl and dimethoate belonged to the same group of compatibility.

Table 3.2: The effect of various insecticides concentrations (means \pm SD) on germination, vegetative growth and sporulation of entomopathogenic fungus *B. bassiana* Strain SGI921.

Treatments	Concentration	Germination %	% Reduction over control	Colony diameter (mm)	% Reduction over control	Fungal spore suspension (spores/ml $\times 10^7$)	% Reduction over control
Control	0%	98.7 \pm 0.58a		18.0 \pm 0.47ed		7.6 \pm 0.22a	
Mospilan	1%	98.3 \pm 0.58ab	0.4	17.5 \pm 0.58def	2.8	7.3 \pm 0.54b	3.9
	2.5%	98 \pm 1.0ab	0.7	17.3 \pm 1.09ef	3.9	7.5 \pm 0.63a	1.3
	5%	97.7 \pm 0.58abc	1	17.4 \pm 0.80ef	3.3	7.4 \pm 0.43ab	2.6
	25%	96.3 \pm 1.53bcd	2.4	17.4 \pm 0.82ef	3.3	3.9 \pm 0.35i	48.7
	50%	95.7 \pm 2.08cde	3.04	17.6 \pm 0.97def	2.2	7.6 \pm 0.26a	0
	75%	94.7 \pm 3.06ed	4.05	18.7 \pm 1.18ab	-3.9	7.4 \pm 0.33ab	2.6
	100%	95.7 \pm 2.52cde	3.04	20.5 \pm 0.846a	-13.8	6.9 \pm 0.31f	9.2
Demeton-s-methyl	1%	97.7 \pm 0.58abc	1	17.9 \pm 0.45e	0.3	7.0 \pm 0.19ef	7.9
	2.5%	97.7 \pm 0.58abc	1	18.1 \pm 0.43de	-0.4	5.6 \pm 0.19g	26.3
	5%	94 \pm 1e	4.8	16.8 \pm 0.42g	6.9	3.4 \pm 0.20i	55.3
	25%	0i	100	16.3 \pm 0.64h	9.7	2.4 \pm 0.25e	68.4
	50 – 100 %	0i	100	0j	100	0m	100
Chlorpyrifos	1%	97.7 \pm 1.53abc	1	18.04 \pm 0.84ed	-0.2	7.2 \pm 0.13cde	5.2
	2.5%	97.7 \pm 0.58abc	1	18.14 \pm 0.54cde	-0.7	7.2 \pm 0.24de	5.2
	5%	98.3 \pm 0.58ab	0	18 \pm 0.91ed	0	6.9 \pm 0.21f	9.2
	25%	94 \pm 2.08e	5	18.2 \pm 0.45cde	-1.1	5.1 \pm 0.17h	32.9
	50%	84 \pm 2.52f	15	17.14 \pm 0.89f	4.8	2.9 \pm 0.26j	61.8
	75%	7 \pm 2.65g	93	16.08 \pm 0.80h	10.7	2.6 \pm 0.2k	65.8
	100%	3 \pm 1h	97	15.25 \pm 0.91i	15.3	2.5 \pm 0.2kl	67.1
Dimethoate	1%	94.5 \pm 3.74e	4.3	17.7 \pm 0.55def	1.7	6.3 \pm 0.45fg	17.1
	2.5%	95.5 \pm 3.41cde	3.3	18 \pm 0.42ed	0	5.1 \pm 0.47h	32.9
	5%	94.5 \pm 3.74ed	4.3	17.7 \pm 0.56e	1.7	4.6 \pm 0.38hi	39.5
	25%	95.3 \pm 2.45cde	3.4	17.8 \pm 0.47e	1.1	4.1 \pm 0.26hi	46.1
	50% - 100%	0i	100	0j	100	0m	100
R-square		0.99205		0.9915		0.995	
C.V		1.95		3.78		3.79	
L.S.D_{0.05}		2.3297		0.37		1.78 $\times 10^6$	

means within a column followed by the same letter does not show significant difference at $\alpha = 0.05$

Table 3.3: Compatibility status of dimethoate, chlorpyrifos, mospilan and demeton-S-methyl with entomopathogenic fungus, *Beauveria bassiana* Strain SGI921 (BI-value)

Treatments	BI- value	Classification
Mospilan		
1%	96.9	Compatible
2.5%	97.5	Compatible
5%	96.9	Compatible
25%	77.3	Compatible
50%	98.60	Compatible
75%	100	Compatible
100%	100	Compatible
Demeton-S-methyl		
1%	95.99	Compatible
2.5%	88.76	Compatible
5%	72.56	Compatible
25%	53.53	Moderately toxic
50%	0	Toxic
75%	0	Toxic
100%	0	Toxic
Dimethoate		
1%	91.72	Compatible
2.5%	85.53	Compatible
5%	81.79	Compatible
25%	79.32	Compatible
50%	0	Toxic
75%	0	Toxic
100%	0	Toxic
Chlorpyrifos		
1%	97.32	Compatible
2.5%	100	Compatible
5%	96.03	Compatible
25%	85.31	Compatible
50%	69.49	Compatible
75%	59.03	Moderately toxic
100%	54.44	Moderately toxic

3.5 Discussion and conclusions

Not all chemical insecticides registered for the control of Russian wheat aphids can be integrated with *B. bassiana* Strain SGI921, in particular. Conidial germination was significantly affected by all insecticides used, in a dose dependent manner. Of the four tested insecticides at various concentrations, Mospilan was the only insecticide that was compatible with the selected strain at all concentrations, even at the recommended application rate of 50g ha⁻¹. Only a slight reduction in germination percentage was observed when the selected strain was allowed to grow on chemically-amended growth media (Table 3.2).

Fungal germination is an important factor of insecticide compatibility evaluation with epf because the initiation of infection is governed by the ability of conidia to germinate on the target host (Anderson and Roberts, 1983; de Oliveira and Neves, 2004; Alizadeh *et al.*, 2007). Moreover, germination tests simulate events in the field, as simultaneous application of insecticides and epf imply direct contact and therefore full exposure to the chemical (de Oliveira and Neves, 2004). Treatment of epf with a chemical may remove the mucous layer covering the conidia, thereby affecting the substrate recognition process and the transduction signal that initiates germination on the target insect (Boucias *et al.*, 1988). Poor germination on fungus treated with insecticide may be the result of organophosphate compounds which directly interfere with cell wall formation through inhibition of an enzyme that converts phosphatidylethanolamine (an aminophospholipid found in cell membranes) into chitin (de Oliveira and Neves *et al.*, 2004).

The results of this study are largely comparable to that of Neves *et al.* (2001) that found *B. bassiana* Strain 447 to be compatible with all tested concentrations of acetamiprid at a field application rate of 25g in 100l ha⁻¹. Germination, colony area and sporulation intensity were only slightly reduced in the presence of this chemical. No significant differences were noted for germination percentage for the control and three tested concentrations. Vegetative growth, on the other hand, was significantly inhibited by acetamiprid at 0.7x, 1x and 1.3x the recommended rate (Neves *et al.*, 2001), while the present study found the 100% rate to enhance radial vegetative growth of SGI921. This implies that the compatibility of chemical and fungi can vary with fungal strains

within the same species. The field application rate in our study was slightly lower (50 g in 300l per hectare) where Neves *et al.* (2001) used 25g/100l. Contrary to our findings, Amutha *et al.* (2010) found acetamiprid to be slightly toxic to *B. bassiana* (strain not stated) using a percentage reduction formula. However, only one field application rate of 500l ha⁻¹ was used and vegetative growth was the only variable determined in that investigation. Sporulation intensity of *B. bassiana* SGI921 was reduced in the presence of acetamiprid at the 1%, 25% and 100% concentrations and were significantly different to the Control (Table 3.2). An inconsistent interaction sometimes exists between fungi and chemicals as shown by 25% Mospilan, huge decline in sporulation intensity was observed and picked up again at 75%. However, the exact mechanism is not known. On the current study, using a biological index formula, all Mospilan doses ranging from 1% to 100% gave rise to biological index values above 70%, which showed compatibility between the two entities.

Chlorpyrifos and demeton-S-methyl significantly reduced conidial germination at ≥ 25 and ≥ 5 %, respectively. Our findings were similar to those of Usha *et al.* (2014), who reported that chlorpyrifos strongly inhibited growth and sporulation of *B. bassiana* at high concentrations (50% and 100% of the recommended rate of 2ml l⁻¹). Nevertheless, their results were strain-dependent because at a low concentration (10%), *B. bassiana* B56 was compatible with chlorpyrifos, whereas with other fungal strains (B44, B55 and B57) the chemical was either highly toxic or moderately toxic, even at low doses. Our study indicated that demeton-S-methyl was toxic at the field recommended rate, resulting in a 100% reduction in fungal activity, while chlorpyrifos was moderately toxic, causing a 15% reduction. Demeton-S-methyl significantly reduced the radial vegetative growth of SGI921, such that 100% reduction was observed at insecticide concentrations ≥ 50 % (Table 3.2). Germination was significantly reduced to 0% at concentrations ≥ 25 %, whereas Raj *et al.* (2011) only found a 46.9% reduction of isolate BBNK007 at the field recommended rate of 3 ml l⁻¹, which is almost the same as used in the current study (3.75 ml l⁻¹).

Dimethoate was found to be toxic to *B. bassiana* SGI921 at concentrations ≥ 50 %. These findings are contrary to what Raj *et al.* (2011) reported. The same insecticide was found to be compatible with *B. bassiana* BBNK007 at half the field application rate and 100% of the recommended dose (0.85ml l⁻¹). However, the application rate of dimethoate was not the same in the two studies: what was considered 25% in our study

was about 100% of what Raj *et al.* (2011) was using. Therefore, in fungal compatibility studies, it is important to note the field application rate used in different cropping systems. Similar to our results, de Oliveira and Neves (2004) found dimethoate to cause about a 90% reduction in *B. bassiana* CG424 germination when 0.5x and full dose was used. Vegetative growth and sporulation intensity of *B. bassiana* CG424 were also significantly reduced in the presence of dimethoate (de Oliveira and Neves, 2004).

Concerning the effect of post-emergence chemical insecticides registered for Russian wheat aphid control on sporulation intensity of *B. bassiana* SGI921, demeton, dimethoate and chlorpyrifos significantly reduced conidial production. The difference in response of cultured fungi might have been due to chemicals present in the insecticide formulations which could also be used as nutrients to increase vegetative growth (Neves *et al.*, 2001). Another possible explanation could be that in a toxic medium the fungus could be making a reproductive effort by increasing conidial production (Raj *et al.*, 2011). The biological index used in the study gave an indication of which combinations should be avoided in integrated aphid control in future. Mospilan was the only insecticide that can be safely used with the Strain SGI921 at the recommended rate. All the emulsifiable concentrates were toxic to a degree to the fungal strains.

Alizadeh *et al.* (2007) and Amutha *et al.* (2010) both found that high toxicity *in vitro* of a given formulation is often matched with a similar level of toxicity under field conditions. Therefore, laboratory assays are an important indication of what is likely to happen under natural conditions. Sub-lethal dosages of chemicals can increase the control efficiency of entomopathogenic fungi as they do not cause adverse effects on the fungi (Schumacher and Poehling, 2012).

In conclusion, all the emulsifiable concentrates registered for Russian wheat aphid control inhibited germination, vegetative growth and conidiogenesis to varying degrees. However, chlorpyrifos dosages less than 25% enhanced the vegetative growth of the fungi even though the increase was not significant. These findings gave an indication of chemical insecticides that should not be integrated with *B. bassiana* SGI921 under field conditions and compatible combinations that should be investigated further under field conditions.

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Chapter 4

Compatibility of the entomopathogenic fungus, *Beauveria bassiana*, with a neonicotinoid and two pyrethrum insecticides against the Russian wheat aphid, *Diuraphis noxia*, on wheat, *Triticum aestivum* cv. Tugela.

4.1 Abstract

The Russian wheat aphid (RWA), *Diuraphis noxia* (Hemiptera: Aphididae), is one of the most destructive aphid pests of wheat grown under dryland conditions in South Africa. When an entomopathogenic fungus (epf), *Beauveria bassiana* (Cordycipitaceae), is applied as a single entity to manage aphids, its efficacy is often challenged by the exponential rate of aphid increase. Hypothetically, the control efficiency of EPFs could be improved if they were used together with synergistic products such as botanical insecticides. Pyrethrum is the main botanical in use, contributing ca. 80% to the global botanical insecticide market. Knowledge of the compatibility between *B. bassiana* and pyrethrum-based insecticides would contribute to the development of an integrated pest management approach against RWA. *Beauveria bassiana* Strain R444 was tested with Pyrol™ (EC) and Xterminator™ (SC) at six concentrations (1%, 5%, 10%, 25%, 50% and 100%) of each insecticide, added to Sabouraud dextrose agar, amended with 1% yeast extract (SDAY). Three parameters were evaluated: germination %, radial vegetative growth and sporulation intensity, in the presence of these insecticides. Germination percentage was not affected at any given concentration by either of the insecticides. Radial vegetative growth ($P < 0.001$, $F_{6,26} = 69.26$, $LSD_{0.05} = 1.340$) and sporulation intensity ($P < 0.001$, $F_{6,26} = 19.77$, $LSD_{0.05} = 2.82 \times 10^6$ conidia ml⁻¹) were significantly reduced at the 50% concentrations and above. A low concentration of Pyrol™ (10%) enhanced sporulation intensity, and the product was therefore selected for further efficacy trials in the glasshouse, together with a 10% dose of acetamiprid (a neonicotinoid insecticide). When comparing the combined products with *B. bassiana* Strain R444 alone, or the control, the Abbot-corrected mortalities were significantly different ($P < 0.0001$ ($F_{7,160} = 181.08$, $LSD_{0.05} = 5.28$)). , although there was no significant difference in mortalities between the control treatment and after application of the recommended dose of a commercial *B. bassiana*-based product, Eco-Bb®. Combined treatments (Eco-Bb® +

10% Pyrol™, R444 + 10% Pyrol™ and R444 + 10% acetamiprid) increased aphid mortality compared to using the fungi and insecticides as individual treatments. Use of epfs combined with with sub-lethal doses of synergistic insecticides could enhance control efficacy, reduce cases of insecticide resistance through reduced selection pressure, reduce pesticide residues in crops and reduce environmental pollution.

4.2 Introduction

Pest management is encountering economic and ecological challenges due to human and environmental hazards by excessive use of synthetic chemicals (El-Wakeil, 2013). As a result, identification of environmentally safe and effective insecticidal products is essential (Sola *et al.*, 2014; Zoubiri and Baaliouamer, 2014). The entomopathogenic fungus (epf), *Beauveria bassiana* (Balsamo) Vuillemin, is registered to control many agricultural pests, including whiteflies, aphids, thrips, weevils and mealybugs (Shah and Goettel, 1999) and it is one of the most used epf in pest management, worldwide (Feng *et al.*, 1994; de Faria and Wraight, 2007).

The Russian wheat aphid, *Diuraphis noxia* (Kurdjomov) (Hemiptera: Aphididae), is a typically r-selected strategist (MacArthur and Wilson, 1967), characterised by a short generation time, small body size and rapid population increase under favourable conditions on susceptible wheat (*Triticum aestivum* L.) (Prinsloo and Tolmay, 2017). When *B. bassiana* is used to manage aphids as a single biocontrol entity, it is often overwhelmed by the nature of the aphid's reproduction strategy. However, the control efficiency of epfs can be improved when used with other control strategies, such as botanical insecticides (Avery *et al.*, 2013). Plants may provide potential alternatives to currently used pest control agents because they constitute a rich source of bioactive chemicals (Wink, 1993). Globally, the demand for plant-based pest control extracts is expected to grow due to an increasing shift in consumer demand for safe food and mounting pressure from new regulations on internationally-traded food (Sola *et al.*, 2014).

Pyrethrum is the main botanical in use, contributing ca. 80% to the global botanical insecticide market (Isman, 2006; Isman, 2017). Pyrethrum's active ingredients (pyrethrins) are three esters of chrysanthemic acid and three esters of pyrethric acid, Pyrethrin I and II are the most abundant, and are responsible for most of the insecticidal activity (Isman, 2006; Cavoski *et al.*, 2011; El-Wakeil, 2013). The mechanism of action

of pyrethrins is characterized by a rapid knockdown effect in flying insects as well as hyperactivity and/or convulsions in most insects (Zibaee, 2011; El-Wakeil, 2013).

Published research on pyrethrum as a bio-insecticide is often based on comparing this botanical insecticide with *B. bassiana*, as opposed to integrating these two bio-pesticides (Castagnolli *et al.*, 2005; Shiberu *et al.*, 2013; Gokturk and Mihli, 2016). In some cases, pyrethrum mixed with other non-chemical agents may also be advantageous. For example, pyrethrum mixed with mineral oil was found to be the most effective aphicide against *Myzus persicae* (Hemiptera: Aphididae), reducing populations from more than 20 aphids per shoot to less than 5 aphids per shoot, outperforming stand-alone applications of the *B. bassiana*-based product BotaniGard® ES (Lo *et al.*, 1999). Nevertheless, several studies have demonstrated that the efficacy of *B. bassiana* can be improved by manipulating host behaviour, integrating it with other control strategies such as sub-lethal doses and mineral oil (Anderson and Roberts, 1983; Neves *et al.*, 2001; Alizadeh *et al.*, 2007). Selected chemicals used to control the Russian wheat aphid, such as demeton-S-methyl, dimethoate and chlorpyrifos, did not show synergistic interactions and were detrimental to the selected fungal strain, SGI921, at the recommended doses (Mzimela *et al.*, 2016 unpublished data).

As pointed out by Islam and Omar (2012), when an IPM strategy is developed, it is important to know the compatibility of epf with other control measures. Most of the published literature on compatibility of *B. bassiana* with botanical insecticides is associated with neem oil (*Azadirachta indica* A. Juss) and on agricultural crops other than wheat (Sahayaraj *et al.*, 2011; Islam and Omar, 2012; Ribeiro *et al.*, 2012; Togbe *et al.*, 2015). However, an innovative formulation based on the combination of natural pyrethrins with *B. bassiana* Strain GHA, called BotaniGard® MAXX, represents the latest innovation in bio-insecticides (BioWorks, 2016). These two biocontrol agents are synergistic and have different modes of action. BioWorks claim that control levels obtained from using BotaniGard® MAXX are comparable to some chemical insecticides. This product suggests that the performance of a South African formulation of *B. bassiana*, Strain R444 (Eco-Bb®), may also be improved by integrating it with natural pyrethrum extract, or a pyrethrum-based product. Pyrethrum has a rapid knockdown effect as opposed to the typical 4-5 days needed by *B. bassiana* to cause mortality (Hatting and Wraight, 2007; Hatting, 2012; Poprawski *et al.*, 2012). The

combination could also increase the target pest spectrum that was originally identified for each individual control strategy. Reducing the amount of active ingredient required to control insect pests will indirectly have a cost-benefit to the bio-insecticide manufacturers. In previous research, we showed that Mospilan was compatible with the strain SGI921, with the highest concentration found to have stimulated vegetative growth. However, since the ultimate goal of the study is to use sub-lethal concentrations of the chemical component, a concentration of 10% was selected to be tested against RWA, also in combination with R444. Neonicotinoids act systematically by travelling through plant tissues in a protein form that is not toxic to the plant, providing effective pest control against sucking insects (Goulson, 2013). Given its proven compatibility with *B. bassiana in vitro*, it was decided to also test the interaction *in vivo*, under glasshouse conditions.

Detailed studies on the compatibility of a commercial formulation of *B. bassiana* R444 with pyrethrum-based insecticides, can assist formulators in optimising Eco-Bb[®]'s efficiency and potentially provide an alternative biocontrol option against Russian wheat aphid. The aim of the study was to identify the concentrations of pyrethrum that are compatible with Eco-Bb[®] and to determine the effect of 10% Mospilan on RWA in combination with Eco-Bb[®].

4.3 Material and methods

4.3.1 *Diuraphis noxia* colony

A clone colony of biotype RWASA1 was established by transferring one adult aphid to wheat plants, cultivar Tugela. Nymphs produced (parthenogenetically) by that single aphid were further reared until the adult stage (about 8 days old) and were then transferred to fresh plants to establish an uncontaminated clone colony. The colony was maintained in a glasshouse at $\pm 23^{\circ}\text{C}$, natural light. Every 2 weeks infested leaves were transferred onto clean plants, allowing the aphids to move from infested to uninfested plants.

4.3.2 Host Plants

Seeds of wheat cv. Tugela were obtained from the Small Grain Germplasm Collection. The selected cultivar was chosen based on its susceptibility to the RWASA1 biotype. Seed germination tests were performed using the “between-paper method” endorsed

by the International Seed Testing Association (ISTA). The actual protocol used in this study was adapted from Rao *et al.* (2006). Briefly, 100 seeds were randomly selected and placed on moisten Anchor seed germination paper (Anchor Paper Co., Minnesota, United States; manufactured by Hoffman Manufacturing, Inc., and supplied by SciCorp Laboratories, Pietermaritzburg, South Africa). Seeds were covered with another sheet of moist germination paper, rolled and tied with a rubber band. Rolls were maintained in large ziplock bags (250 mm x 320 mm, supplied by Glad Company, <http://www.glad.co.za/prod-FOOD-zipSeal.php>). Ziplock bags were kept in an incubator at 22°C, total darkness, for four days prior to planting. Germinated seeds were planted into plastic seedling trays 300 mm x 270 mm x 105 mm containing sterile and fertilized soil and seedlings used in the bioassays at the 4-leaf stage.

4.3.3 Biocontrol agents

Beauveria bassiana Strain R444 was obtained from the National Collection of Fungi, ARC-Plant Health and Protection, Pretoria, but originally isolated from soil in the Western Cape, South Africa (Hatting *et al.*, 2004). The chosen isolate is the active ingredient in a commercial formulation of *B. bassiana* (Eco-Bb® also known as Bb-Protec®). Eco-Bb® is registered by the company Plant Health Products (Pty) Ltd., under Act 36 of 1947, for the control of whitefly and suppression of red spider mite on beans, tomatoes, cucumbers and brinjals (PHP, 2017). The company has also been granted an emergency registration of Eco-Bb® against fall armyworm on maize, sweetcorn and other crops (PHP, 2017). The fungus was passed, twice, through larvae of the greater wax moth, *Galleria melonella* L. (Lepidoptera: Pyralidae), before being cultured at 23°C, total darkness, on Sabouraud dextrose agar amended with 1% yeast extract (SDAY). Conidia were selectively collected from 14-day old cultures with confirmed conidial viability of ≥95% germination (after 24h incubation). A conidial suspension of 1×10^8 conidia ml⁻¹ was prepared in 0.01% Break-Thru® surfactant and quantified using a Neubauer haemocytometer.

One gram of commercial formulation of *B. bassiana* (Eco-Bb® WP), (min. 2×10^9 conidia per gram) was added to 1L sterile water as per product label.

4.3.4 Insecticides

Three insecticides were used in the study, Pyrol EC, Xterminator™ SC and Mospilan 20 SP. Pyrol EC is a certified organic contact insecticide registered to control aphids, with active ingredients: canola oil 895 g l⁻¹ and pyrethrins 5 g l⁻¹. This pyrethrum-based insecticide was obtained from Grow Guru Horticulture (Pty) Ltd, Port Elizabeth, South Africa. Xterminator™ SC is a contact and stomach insecticide supplied by Agro-Organics (Pty) Ltd, Cape Town, South Africa. Active ingredients: pyrethrins 75 g l⁻¹, rapeseed oil 300ml l⁻¹, citrus oil 400ml l⁻¹ and soft soap 290ml l⁻¹. Mospilan 20 SP was selected on the basis of being compatible with *B. bassiana* SGI921 (*cf.*, Chapter 3).

4.3.5 Compatibility of pyrethrum-based insecticides with *Beauveria bassiana*

Six concentrations (1%, 5%, 10%, 25%, 50% and 100%) of each insecticide were added to SDAY, cooled to *ca.* 40°C, to test compatibility with *B. bassiana* Strain R444. Thereafter, 10 µl of 5 x 10⁷ conidia ml⁻¹ of R444 was inoculated at the centre of a 65mm Petri dish with SDAY amended with various products at the different concentrations. Germination percentage was assessed at 24 hours post inoculation by microscopically observing (40x magnification) 100 randomly selected conidia from different fields of view; radial vegetative growth was measured after 7 days and conidial production (conidiogenesis) was assessed 9 days after incubation according to the protocol of Ribeiro *et al.* (2012).

4.3.6 Greenhouse trials testing selected concentrations of Pyrol and Mospilan with formulated and unformulated conidia of *Beauveria bassiana*

Ten adult aphids were collected from the RWA clone colony (see section 4.3) and placed individually on ten aphid-free wheat plants, cultivar Tugela, at the 4 leaf stage. After 24 hours, adult aphids were removed and the remaining nymphs reared until the adult stage for use in the assay. The aphids were inoculated using the triangle method adapted from Hatting and Wraight (2007). Suspension aliquots of 5ml per treatment were topically applied onto adult RWA placed on prepared triangles inside a Burgerjon spray tower (see Chapter 2, section 2.3.4 and 2.3.5). After inoculation, aphids were allowed to move onto the clean plants. The experiment consisted of a control (0.01% BreakThru), *B. bassiana* Strain R444 (5 x 10⁷ conidia ml⁻¹), Eco-Bb (1g l⁻¹), Pyrol (40ml l⁻¹); recommended application rate), 10% Pyrol, 10% Mospilan, R444 + 10% Pyrol, Eco-Bb® + 10% Pyrol and R444 + 10% Mospilan). Mospilan-treated plants were

sprayed with a hand-held sprayer (30 ml. m⁻², which equals an application rate of 300l ha⁻¹), 24hours prior to infestation with aphids. Each treatment was replicated 5 times, *i.e.*, 5 pots with 20 aphids each. Mortality was recorded every 24 hours for a period of 7 days after treatment. Dead aphids were transferred to water agar to determine overt mycosis.

4.3.7 Statistical analyses

A one-way ANOVA was performed with various concentrations of bioinsecticides for both radial vegetative growth and sporulation intensity of the fungus. The experimental design was a randomized complete block with three replicates with 15 and 5 sub-samples for the vegetative growth and sporulation intensity, respectively. The same analysis was used for the glasshouse mortality data, with three replicates, with 9 treatments and 5 sub-samples for each treatment. The standardised residuals were normally distributed (Shapiro-Wilks test) and therefore the means of the significant effects were separated using Fisher's Unprotected t-test (least significant difference – LSD) tested at the 5% level of significance (Snedecor and Cochran, 1980). All data analysis were performed using SAS 9.4 statistical software (SAS, 2014).

Each trial was repeated three times and data were pooled. Abbott's formula was used to correct for control mortality (Abbott, 1925), before subjecting mortality data of RWA to analysis of variance.

4.4 Results

The 7-day evaluation of radial vegetative growth was not possible as the plates were overrun by cultures of *Aspergillus* spp. and *Trichoderma* spp and germination percentage of Eco-Bb[®] was too low to count because recommended dose could not show any fungal spores on agar plates.

4.4.1 Compatibility of pyrethrum-based insecticides with *Beauveria bassiana* (Germination)

The germination test determined the number of conidia that germinated from 100 conidia, selected randomly, on an inoculated plate. Percentage germination was not affected by Pyrol, with an average 96.7±3.1% overall germination measured across all concentrations, even with 100% Pyrol (Table 4.1). In addition, the average length of

the conidial germ tube was also used to determine the effect of the bioinsecticide on the selected fungal strain. At the higher concentrations, the average germ tube lengths were slightly shorter than the control germ tubes and the mycelium was less dense than the control mycelium (Figure 4.1). Judging from the percentage germination and germ tube lengths, *B. bassiana* was considered compatible with Pyrol.

High concentrations of Xterminator affected some parameters such as radial vegetative growth and sporulation intensity. However, the fungus was initially not affected by the presence of the chemical (24hours post inoculation), with a high level of germination even at 100% concentration of Xterminator (Table 4.1). Notably, after seven days, the bioinsecticide reduced both vegetative growth and conidiation (Figure 4.2).

Table 4.1: The effect of Pyrol and Xterminator on germination percentage \pm SD of *Beauveria bassiana* R444 Strain after 24 hours.

Treatments	Pyrol	Xterminator
Control		98.8 \pm 1.2
1%	98.7 \pm 2.0	98.0 \pm 2.8
5%	95.3 \pm 5.8	98.3 \pm 1.2
10%	97.7 \pm 1.5	96.8 \pm 4.3
25%	98.2 \pm 1.7	97.2 \pm 3.2
50%	97.5 \pm 2.6	97.5 \pm 1.6
100%	98.2 \pm 3.1	94.7 \pm 5.2

When compatibility of Xterminator with *B. bassiana* Strain R444 was evaluated using the length of germ tubes, clear visual differences were observed. The photos were taken with Olympus BX53F digital Microscope, Made in Japan (supplied by Wirsam Scientific, Johannesburg, South Africa).

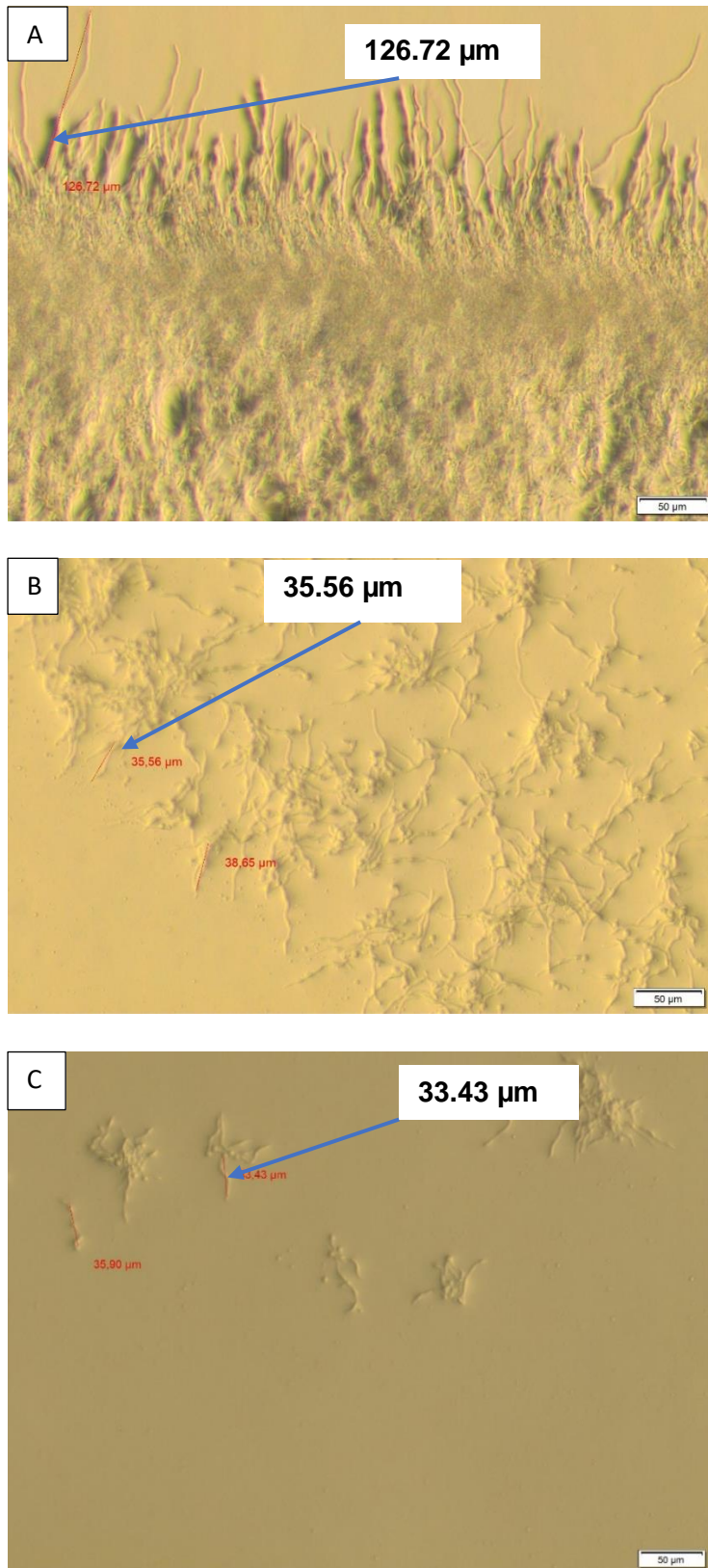


Figure 4.1: Comparison of germ tube length of *Beauveria bassiana* Strain R444 inoculated on, (A) chemical-free growth media vs. (B) half (50%) and (C) full strength (100%) recommended doses of Xterminator.

4.4.2 Evaluation of radial vegetative growth of *B. bassiana* Strain R444 on SDAY medium treated with Pyrol and Xterminator, pyrethrum-based insecticides.

The bioinsecticides caused significant reductions in radial growth of *B. bassiana* Strain R444 ($P < 0.001$, $F = 140.98$). Pyrol, at the 50 and 100% concentrations, and Xterminator at 25% concentration and above, were inhibitory. The rest of the treatments did not significantly affect the fungus (Figure 4.2).

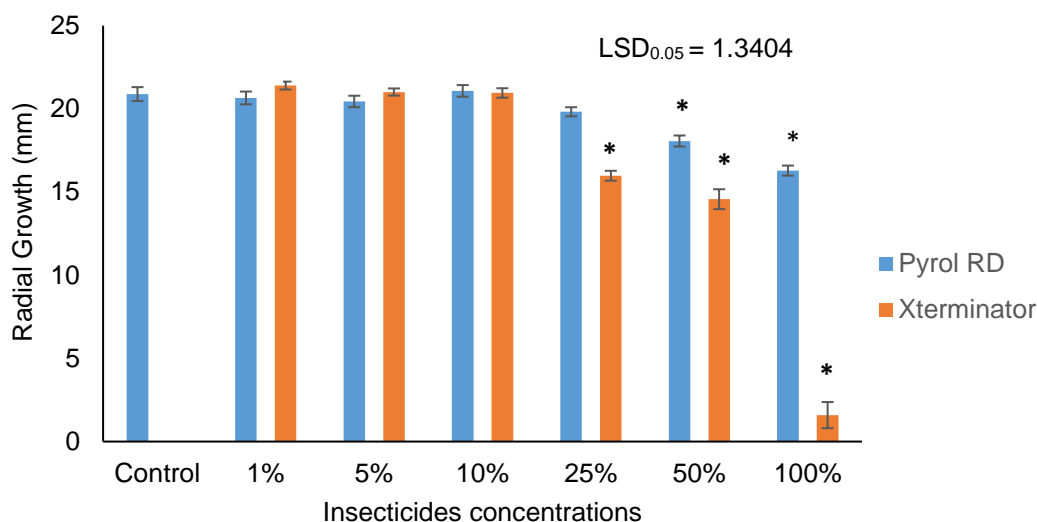


Figure 4.2: Radial vegetative growth of *Beauveria bassiana* Strain R444 exposed to different concentrations of Pyrol and Xterminator insecticides on SDAY medium, 7 days post inoculation. Significance at the 5% test level indicated by *.

4.4.3 Evaluation of sporulation intensity of *B. bassiana* Strain R444 on SDAY medium treated with Pyrol and Xterminator, pyrethrum-based insecticides.

When sporulation intensity of *B. bassiana* R444 was tested in the presence of pyrethrum-based insecticides, a linear relationship was obtained (Figure 4.3). The interaction between insecticide and concentration was found to be significant ($P < 0.001$, $F = 22.01$). Pyrol at 10%, enhanced the production of conidia of *B. bassiana* Strain R444. Higher concentrations of Pyrol had inconsistent interactions. Xterminator significantly reduced sporulation, even at a 5% concentration. The best performing product x concentration (10% Pyrol) was then selected for glasshouse trials against Russian wheat aphid, RWASA1.

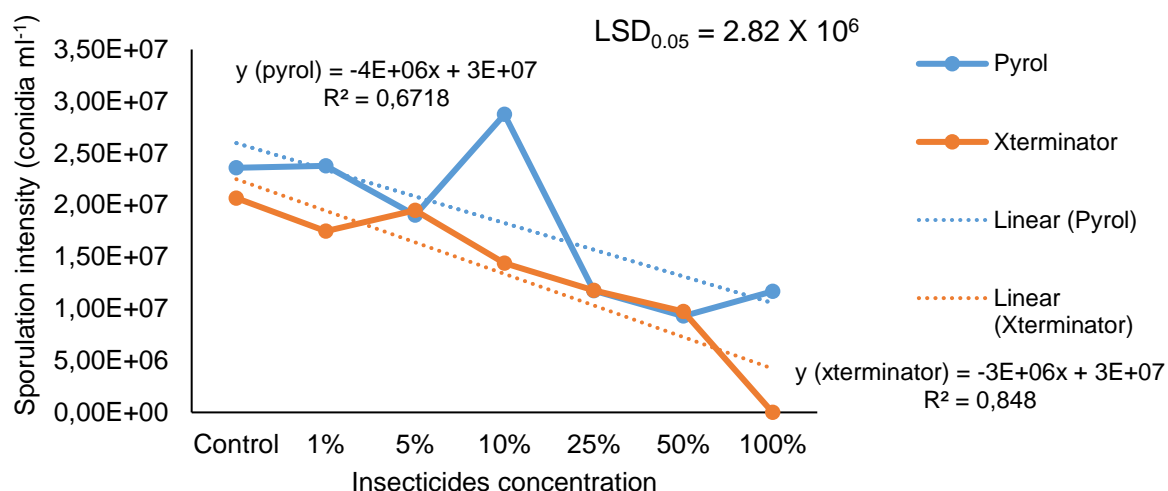


Figure 4.3: Sporulation intensity or conidiation of *Beauveria bassiana* R444 in the presence of pyrethrum-based insecticides at various concentrations.

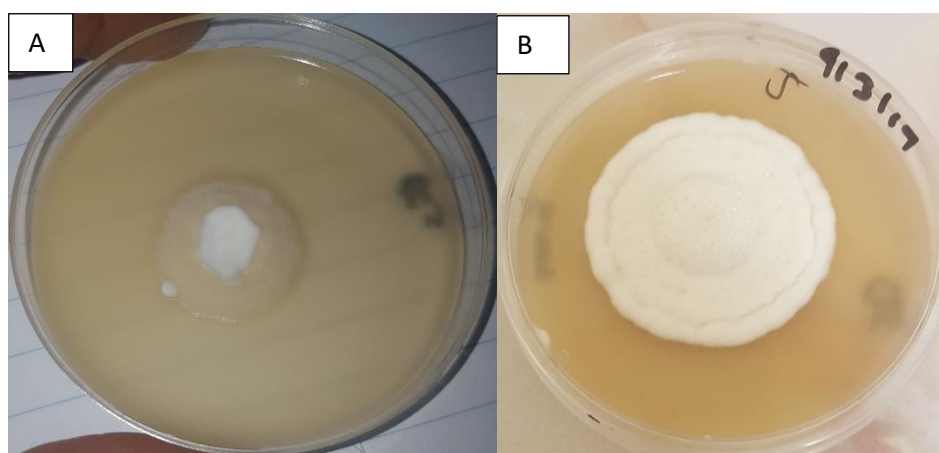


Figure 4.4: An inhibited *Beauveria bassiana*, R444 colony (A) exposed to 25% Pyrol concentration, compared to the control (B) at 15 days post inoculation.

Even though selected insecticides were found to be compatible with *B. bassiana* at some concentrations, individual cultures were not able to tolerate the insecticide 15 days post-inoculation, as shown in the example in Figure 4.4. Zones of clearance were shown by a creamy colour that had lost the original *B. bassiana* colour.

4.4.3 Interactions between entomopathogenic fungi and pyrethrum-based bioinsecticides against *D. noxia*.

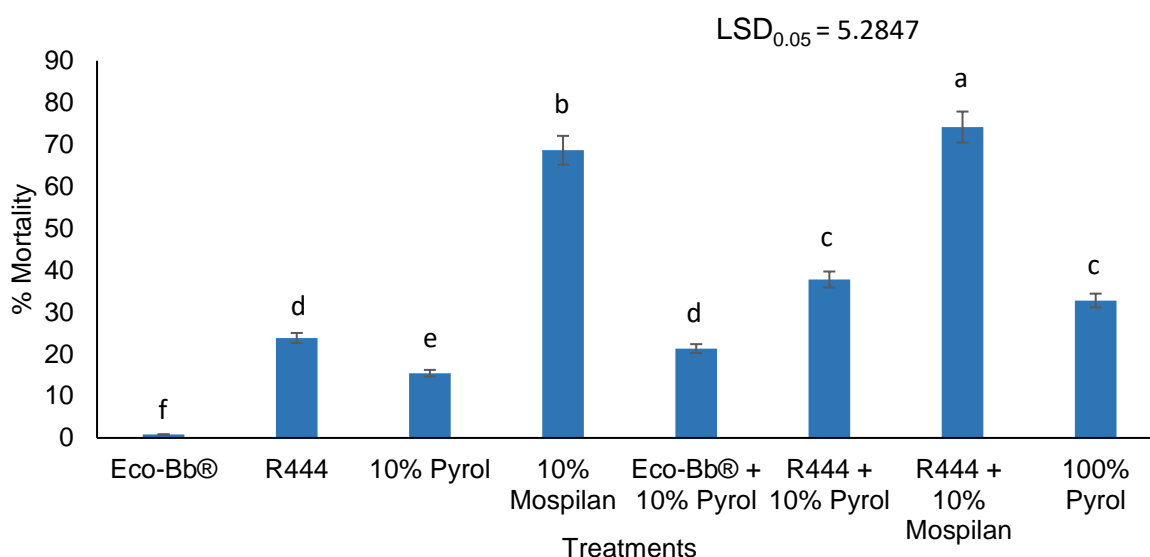


Figure 4.5: Abbot-corrected mortality of *Diuraphis noxia* exposed to formulated and unformulated *Beauveria bassiana* Strain R444, combined with a sub-lethal dose of pyrethrum and Mospilan insecticides, or as solo products. Bars with different letters, differed significantly at the 5% test level.

Abbott-corrected mortalities showed that treatments differed at $\alpha = 0.05$ ($P < 0.0001$, F-value = 181.08). Reduced doses (10%) of Pyrol and Mospilan, caused 15% and 69% Abbott-corrected mortalities, respectively. However, by adding the fungal component to Pyrol, both the commercial product (Eco-Bb®) and pure conidia (R444), significantly enhanced its efficacy to 21.3% and 37.7%, respectively. Also for 10% Mospilan (68.6% mortality), a significant improvement in efficacy was noted when used in combination with R444 (fungal conidia), increasing mortality to 74%. The combined performance of Eco-Bb® + 10% Pyrol, statistically outperformed the solo treatments of both 10% Pyrol and Eco-Bb®. Although not statistically different, R444 + 10% Pyrol caused slightly higher mortality than the 100% Pyrol treatment. Highest mortality (74%) was observed with a combination of R444 + 10% Mospilan, statistically outperforming all other treatments.

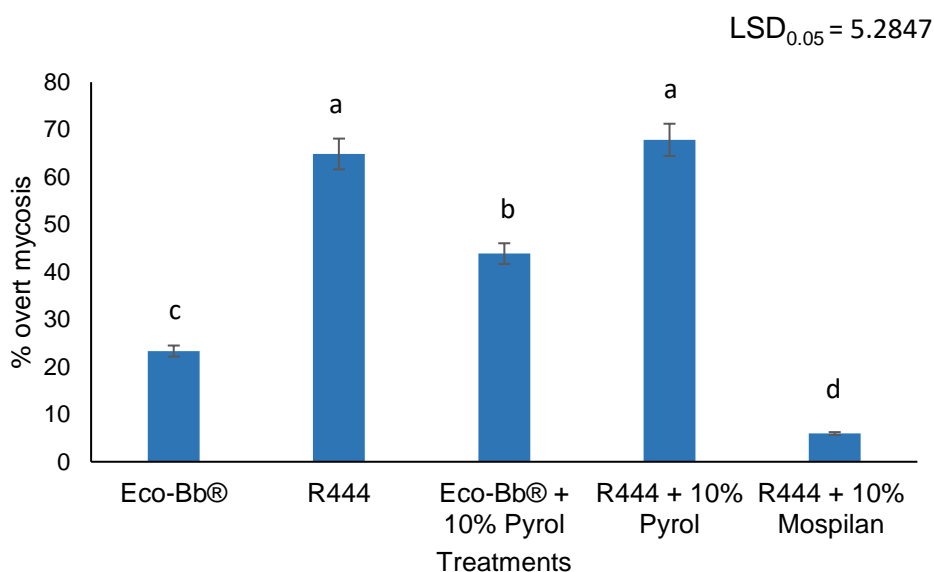


Figure 4.6: Percentage overt mycosis on *Diuraphis noxia* cadavers treated with formulated, Eco-Bb® and unformulated *Beauveria bassiana* strain, R444 integrated with a sub-lethal dose of pyrethrum and Mospilan insecticides. Bars with different letters, differed significantly at the 5% test level.

Combined products had higher overt mycosis as opposed to a single control agent ($P < 0.001$, $F = 69.26$, $LSD_{0.05} = 9.897$). For example, R444 and Eco-Bb® caused 64% and 23% average overt mycosis as solo treatments, respectively; whereas highest overt mycosis (67%), was observed with integration of R444 + 10% Pyrol but not significant different with R444 at 5% level.

4.5 Discussion and conclusion

Liu (2012) noted that conidial germination is the most vulnerable part of the *B. bassiana* lifecycle (initiation of infection) and therefore, the best growth stage to monitor for the effects of chemical and botanical insecticides. Another variable, “*in vitro* persistence”, should be added in future research because this study showed that samples that were compatible at early stages of development, radial vegetative growth (Figure 4.2) were not viable at 15 DPI (Figure 4.4)

Compatibility studies have the advantage of exposing fungi to the maximum action of the chemicals, which does not necessarily occur under field conditions, because of other biotic and abiotic factors (Hirose *et al.*, 2001). This was the first scientific study

to determine the effect of pyrethrins on *B. bassiana* R444. Much research has been conducted on the compatibility of *B. bassiana* with the botanical insecticide Neem (neem oil, Azadirachtin) (Depieri *et al.*, 2005; Mohan *et al.*, 2007; Ambethgar, 2009; Amutha *et al.*, 2010; Ribeiro *et al.*, 2012), but little has been done with natural pyrethrins. Anderson and Roberts (1983) found synthetic pyrethroids (permethrin and fenvalerate) to be toxic to six *B. bassiana* isolates that were obtained from France (Strain 149), USSR (220, 288) and USA (252, 308, and 338). Perez-Gonzalez and Sanchez-Pena (2017) found similar results with another synthetic pyrethroid, cypermethrin, when it was tested at field application rates. It slightly inhibited vegetative growth of three *B. bassiana* isolates (BbDc34, BbYs35 and BdLourdes), but their germination was not affected and these results are comparable to that of the current study. Lastly, de Oliveira *et al.* (2003) recommended the combination of alpha-cypermethrin and *B. bassiana* Strain CG425 for the control of coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae), since the pair was found to be compatible at half the recommended application rate. These findings were based on synthetic pyrethroids, which share similarities with natural pyrethrins, such as mode of action, even though constituents' differ.

Even though Xterminator was found to be toxic at high concentrations to the selected fungal strain, Amutha *et al.* (2010) stated that the same reaction might not prevail under field conditions and therefore integration of the two agents cannot be completely ruled out.

A major component in Pyrol, is canola oil. Vegetable oils often enhance the effect of mycopathogens by facilitating conidial adhesion and germination and by extracting fungistatic compounds from the insect cuticle (Mohan *et al.*, 2007). As a result, the observed interactions could result from the canola oil as opposed to the actual active ingredient, pyrethrins. Therefore, the study can be extended further by sourcing the pyrethrum extract and/or conduct bioassays on the effect of canola oil alone on *B. bassiana*.

Aphid mortality caused by the commercial biocontrol formulation, Eco-Bb®, was not significantly different to the control. One of the possible reason could be that *B. bassiana* spores do not easily attach and germinate on healthy aphids (Laing, 2017 personal communication). The exact mechanisms responsible for the interaction

between fungal spores and cuticle was beyond the scope of this research. However, Valero-Jimenez *et al.* (2016) reported that successful invasion requires that fungal propagules overcome host immune response. The compositions of cuticle and conidial surface also influence adhesion potential. Since the *B. bassiana* infection process involves different stages, it is important to determine the exact stage where the infection process was halted. The use of advanced molecular technologies to track fungal pathways during infection should resolve the speculations. Castrillo *et al.* (2005) mentioned that conidia landing on an insect cuticle may not produce any reaction because of an absence of recognition between the fungus and insect host. The inability of fungi to penetrate the cuticle is associated with the presence of inhibitory compounds such as phenols and lipids that are on the cuticular surface (Hajek and St. Leger, 1994). Lastly, Zhang *et al.* (2008), found that adding yeast-expressed Pr1 protease CDEP-1 into the fungal suspension increased conidial germination of *B. bassiana* Bb0062, thus improved its virulence against green peach aphid, *M. persicae*. Genetic or biochemical modification of *B. bassiana* R444 could enhance the virulence of Eco-Bb®.

Knowing that aphids are not amongst the target pests that this product is registered for, could explain this low mortality, but assessing the product in the laboratory showed other contaminants that could hinder *B. bassiana* establishment.

Mospilan at 10% (0.016g l⁻¹) was effective against RWA, killing almost 70% of the aphids (Figure 4.5). The same pattern was observed when it was combined with R444. Xiao *et al.* (2015) found that the LC₂₅ for pirimicarb against *R. padi* and *S. avenae* were 0.29 µg ml⁻¹ and 0.60 µg ml⁻¹, respectively. These concentrations resulted in corrected mortalities of 25.8% and 23.2% for *R. padi* and *S. avenae*, respectively. The concentrations tested in the current study were higher, and killed instead of stressing the aphids. Future research could test a concentration of 1% or even lower to obtain sub-lethal doses.

Neonicotinoids are highly effective insecticides for the control of sucking insect pests such as aphids (Elbert *et al.*, 2008). Published research on *in vivo* compatibility of *B. bassiana* with imidacloprid was also reported on other insect pests such as silverleaf whitefly, *Bemisia argentifolii* Bellows and Perring (Homoptera: Aleyrodidae) (James and Elzen, 2001), citrus root weevil, *Diaprepes abbreviatus* Linnaeus (Quintela and

McCoy, 1998) and Colorado potato beetle *Leptinotarsa decemlineata* Say (Furlong and Groden, 1991) on cultivated crops other than wheat. In the case of RWA, the 10% dose was enough to cause 68.6% corrected mortality. Sublethal doses of acetamiprid, combined with entomopathogenic fungi, could slow the development of resistance in other cereal aphids such as *R. padi*. Zuo *et al.* (2016) reported this aphid species has developed some resistance to acetamiprid alone. Al-Mazra`awi (2006) also tested interaction effects between *Beauveria bassiana* and imidacloprid against *Thrips tabaci* (Thysanoptera: Thripidae). The results are comparable to the current study, because 10% imidacloprid caused 83% mortality, *B. bassiana* GHA (52%) and combined effect resulted to higher mortality, 85% (GHA + 10% imidacloprid). Clavet *et al.* (2013) also found that combination of 10% *B. bassiana* GHA with a field recommended rate of imidacloprid, also increased annual bluegrass weevil, *Listronotus maculicollis* Kirby (Coleoptera: Curculionidae) mortality within 24 hours post application. Synergistic interactions between *B. bassiana* and imidacloprid could be time dependent, because combination of *B. bassiana* formulation (Biosoft) with imidacloprid, significantly reduced the number of mites, *Tetranychus urticae* Koch (Prostigmata: Tetranychidae), only at 5 days post inoculation and was not significant at 7, 9 and 11 DPI (Gupta, 2015). Morales-Rodriguez and Peck (2009), also reported synergistic interactions between *B. bassiana* (Botanigard ES) and imidacloprid at 50% and 25% of the recommended rate in white grubs, *Popillia japonica* Newman (Coleoptera: Scarabaeidae). Antagonistic interactions between *B. bassiana* and imidacloprid also occurs, as shown by James and Elzen (2001), in *Bemisia argentifolii* (Homoptera: Aleyrodidae) control. Such phenomena should be taken into account when considering combination treatments.

The highest mortality was recorded within 48 hours, and the speed of action indicates that the aphids were killed by Mospilan. Overt mycosis results confirmed that only 6% were killed by the inoculated strain when Mospilan was combined with R444 (Section 4.4.3).

The fungal strain used in the study caused a corrected mortality of 23.8%, at 7 days post inoculation. This result is different from those reported in Chapter 2 where R444 caused a 51.5% mortality. Literature on fungal pathogenesis has emphasized that one of the major drawbacks of using fungi alone is their inconsistency. Combined treatments (Eco-Bb® + 10% Pyrol and R444 + 10% Pyrol) were found to have synergistic effects on aphids' mortality (Figure 4.5). Mohan *et al.* (2007) obtained

similar results with neem oil, Margoside® applied in the field at the recommended rate, when combined with about 30 *B. bassiana* isolates against *Spodoptera litura* Fabricius. Farenhorst *et al.* (2010) also found that combining *B. bassiana* with permethrin reduced mosquito survival more than using these control agents alone.

The commercial product, BotaniGard® MAXX, has been registered because combining *B. bassiana* with pyrethrin can result in synergistic effects. Mortality of RWA caused by 100% Pyrol was not significantly different to R444 + 10% Pyrol (Figure 4.5). Reducing the required concentration can be cost efficient, because instead of using 40ml per litre in one spray application, farmers could use 4ml l⁻¹. However, this strategy must be tested on other insect pests and agricultural crops in order to confirm what was found on RWA. Sub-lethal dose combinations do not only result in a cut-costing strategy, but reduce levels of resistance in target pests, minimize cases of environmental pollution and also reduce the maximum residue levels on harvested crops, and can also be sprayed a few days prior to harvest.

A possible drawback of using entomopathogenic fungi plus sublethal doses of insecticides could be failure of biological component to survive adverse conditions in the field, for example dry out immediately after application and thus lose benefit of synergism. Lastly, if target insects are only exposed to sublethal doses without fungi, there is a risk of development of resistance. Therefore, time of application and field conditions should favour fungal germination for success of the strategy.

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An Overview of research findings

Introduction and objectives of the study

South Africa is a net importer of wheat and depends on imports to supply the growing local demand of wheat (SAGL, 2016). Although, the area planted with wheat increased slightly by 1.2% in the 2015/2016 growing season, the mean national yield declined from 3.67 t ha⁻¹ (2014/2015) to 2.99 t ha⁻¹ (2015/2016). The commercial crop of wheat produced in the 2015/2016 season was also 17.7% lower than the previous season (SAGL, 2016). This trend of decreasing wheat production results in increased imports. It is difficult for South African farmers to produce wheat profitably and they have slowly switched to more profitable crops such as canola, maize and soybean (USDA, 2013). Since global demand for food is expected to increase by 98% by 2050, the situation will be even worse and therefore farmers will need support to increase crop production by enhancing productivity on existing agricultural land.

The Russian wheat aphid, *Diuraphis noxia* is the most economically important aphid pest of cultivated wheat in South Africa, causing serious damage annually (Du Toit and Walter, 1984; Du Toit, 1992; Tolmay, 2006). Host plant resistance to RWA is generally seen as an effective, environmentally responsible, economically and socially acceptable method of pest control, which plays a fundamental role in sustainable agricultural systems (Tolmay, 2006; Pathak *et al.*, 2007; Porter *et al.*, 2009). However, resistant breaking biotypes continue to pose a serious threat to wheat producers in the country (Jankielsohn, 2017) and thus renders the method less effective. Periodic droughts known to occur in the Free State region make it difficult to achieve effective chemical control of RWA under these dry conditions (Du Toit, 1992).

Entomopathogenic fungi (epf), such as *Beauveria bassiana* have the ability to suppress RWA as shown by (Hatting *et al.* (2004). When *B. bassiana* Strain GHA was combined with host plant resistance, control levels of ca. 65% were recorded. Different methods have been developed to enhance the efficiency and insect mortality by combining epf with sub-lethal doses of chemical insecticides, and the resulting interactions can range from being synergistic, antagonistic to neutral (Sahoo and Dangar, 2014). Synergistic effects would reduce the amount of insecticide, minimize

environmental pollution, and preserve natural enemies (Ambethgar, 2009). Chemical insecticides act as a stress inducer and make the insect pest to be more susceptible to fungal infection (Benz, 1971; Ambethgar, 2009; Malekan *et al.*, 2012).

This study aimed to enhance the virulence of selected entomopathogenic fungal strains through synergism with sub-lethal doses of chemical and/or botanical insecticides.

The objectives of the study were:

- To find several virulent entomopathogenic strains of *B. bassiana* and *M. anisopliae* against *D. noxia*
- To screen insecticides, registered for RWA control, for compatibility with selected fungal strains in terms of germination, radial vegetative growth and sporulation.
- To determine the effect of combining sub-lethal doses of pyrethrum-based insecticide with a commercial formulation of *Beauveria bassiana*, Eco-Bb[®], and *Beauveria bassiana* Strain R444, *in vivo* and *in vitro*.

Research findings in brief and recommendations for future research

Pathogenicity of isolates of *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin against the Russian wheat aphid, *Diuraphis noxia* (Hemiptera: Aphididae)

Three isolates of *Beauveria bassiana* and three of *Metarhizium anisopliae* were screened for virulence against *Diuraphis noxia*, RWASA1 biotype. The aphids were sprayed with a suspension of 5×10^7 conidia ml⁻¹, which resulted to about 1000 conidia per mm², and were then kept in a controlled environment for 7 days to monitor and record mortality. Three variables were evaluated in the study, mortality (%), overt mycosis and mean time of mortality.

- All six fungal isolates were found to be pathogenic to *D. noxia*, whereby the lowest mortality was 31%. The level of their virulence varied between species and strains within species.

- The most virulent species was *B. bassiana* since it caused highest Abbott-corrected mortality, counts of overt mycosis and lastly, the speed with which aphids were killed, the LD₂₅.
- *B. bassiana* Isolate SGI921, was the most pathogenic strain, and it could be considered for further investigation as a biocontrol agent against RWA.

Future studies should focus on screening more than three *B. bassiana* strains to quantify their virulence against RWA. Further concentrations of conidia will also be tested in order to calculate the LC₅₀ values.

***In vitro* compatibility between selected chemical insecticides and *Beauveria bassiana* strain SGI921**

The aim of the study was to test the *in vitro* compatibility of *Beauveria bassiana* Strain SGI921, with four chemical insecticides (active ingredients: chlorpyrifos, dimethoate, demeton-S-methyl and acetamiprid). The impact of these insecticides on the fungus was evaluated using germination percentage, radial vegetative growth and sporulation intensity.

- All the tested insecticides reduced germination, radial vegetative growth and sporulation intensity of the selected strain to various degrees in a concentration-dependent manner.
- According to a biological index for the classification of the toxicological effect of chemical compounds on entomopathogenic fungi, all tested emulsifiable concentrates (chlorpyrifos, demeton-S-methyl and dimethoate) ranged from moderately toxic to toxic for concentrations of 50% and above.
- Emulsifiable concentrates had the most antagonistic effects and at these concentrations they should not be used in chemical-fungal combinations.
- Mospilan (acetamiprid) was found to be compatible with the selected strain and only the highest concentration seemed to have stimulated vegetative growth.

Future research will focus more on concentrations below 10% since higher concentrations were detrimental to fungal performance. Additional work will assess more than one *B. bassiana* strain or other fungal species to validate the findings.

Compatibility of the entomopathogenic fungus, *Beauveria bassiana*, with pyrethrum against the Russian wheat aphid, *Diuraphis noxia*, on wheat, *Triticum aestivum* cv. Tugela.

The study evaluated the effect of combining *B. bassiana* with two pyrethrum-based insecticides, Biogrow Pyrol and Xterminator *in vitro*. These were also tested for the effect of sub-lethal doses of Pyrol combined with *Beauveria bassiana* (unformulated conidia of R444 and formulated Eco-Bb®), in the glasshouse against RWA. Mospilan at a concentration of 10% was also added in the glasshouse trial, based on its compatibility with *B. bassiana*, as reported in Chapter 3.

- Xterminator was found to be detrimental to fungal growth at concentrations above 25% and as a result, only 10% Pyrol was selected for glasshouse efficacy trials.
- The combined performance of Eco-Bb® + 10% Pyrol, statistically outperformed the solo treatments of both 10% Pyrol and Eco-Bb®.
- For 10% Mospilan (68.6% mortality), a significant improvement in efficacy was noted when used in combination with R444 (fungal conidia), increasing mortality to 74%.
- Sub-lethal dose combinations have an ability to enhance control efficacy, reduce cases of insecticide resistance and reduce the time to kill by the biocontrol agents, and to reduce environmental pollution.

Future research will focus on testing the active ingredient in Pyrol since the actual synergist might be constituents other than natural pyrethrins. Another option would be to test the effect of canola oil on *B. bassiana*. Combination of sub-lethal doses of insecticides will also be tested on other cereal aphid species (e.g., *Rhopalosiphum padi*, an important vector of Barley Yellow Dwarf virus) and non-target organisms to ensure safety of the product.

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